

COVALENT MODIFICATION OF ENZYMES FOR TEXTILE PROCESSES

by

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COVALENT MODIFICATION OF ENZYMES FOR TEXTILE PROCESSES

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to my family and Tekin,

ABSTRACT

Wide range of application of enzymes allowed their use in many textile processes such as biopolishing, desizing and bleaching. In this work the focus was on two separate applications of modified enzymes in textile processing; retaining strength of viscose during pilling process and combining desizing and bleaching processes.

Cellulases are used to prevent pilling of the viscose fabric but they cause a loss in the tensile strength of fabric. Commercial cellulases were crosslinked using different parameters to ameliorate loss of tensile strength and improve enzyme properties. Native and modified enzymes were characterized and their activities against CMC and their effects on the properties of viscose (such as pilling, bursting strength) were determined. Effects of mechanical agitation and surfactants were examined. Crosslinking of cellulase was found to prevent the loss of strength because larger enzyme complexes formed by crosslinking were mainly restricted to the fabric surface.

Enzymes are replacing use of harsh chemicals in many of the textile processes and immobilization of enzymes on solid supports allow their recycling. Enzymatically produced peroxide is used for bleaching of cotton fabrics. Commercial Glucose oxidase (GOx) was immobilized on different supports. Crosslinked enzyme aggregates (CLEA) of GOx were prepared. Their efficiencies against glucose for peroxide production were analyzed. Moreover, starch size on cotton fabric is hydrolyzed into glucose by amyloglucosidase and this liquor was used to produce peroxide. Activities of immobilized enzymes and CLEAs against desizing liquor and whiteness values of cotton fabrics after bleaching were examined. Whiteness values reached with immobilized enzymes are not appropriate for white textiles but are sufficient for further dyeing processes. Combination of desizing and bleaching in a single bath and recycling of the immobilized enzyme is an environmentally friendly alternative.

ÖZET

Enzimlerin geniş uygulama alanları, onların biyoparlatma, haşıl sökme ve ağartma gibi pek çok tekstil işleminde kullanılmalarına olanak sağlamaktadır. Bu çalışmada, değişikliğe uğratılmış enzimlerin tekstil terbiyesinde iki farklı uygulamasına odaklanılmıştır. Bunlardan ilki viskon kumaşta biyoparlatma işlemi sırasında görülen kumaş mukavemet kayıplarını önlemek, diğeri ise haşıl sökme ve ağartma işlemlerini birleştirerek işlem verimini arttırmaktır.

Selülozlar viskon kumaşlarda tüylenmenin önlenmesinde kullanılmaktadır ancak aynı zamanda viskon kumaşın mukavemetini düşürmektedir. Enzim özelliklerini geliştirmek ve mukavemet kayıplarının üstesinden gelmek üzere ticari selülozlar çapraz bağlanmıştır. Ham ve değişikliğe uğratılmış enzimler karakterize edilmiş ve karboksimetilselüloza karşı aktiviteleri ile viskon kumaşa olan etkileri belirlenmiştir. Mekanik ajitasyonun ve çeşitli yüzey aktif maddelerin enzimlerle birlikte kumaş yüzeyine etkilerine bakılmıştır. Çapraz bağlamayla oluşan daha büyük enzim ağlarının esas olarak kumaş ve lif yüzeyinde kalmaları sebebiyle, viskonda biyoparlatma esnasında görülen mukavemet kayıplarını önlediği bulunmuştur.

Enzim kullanımı, haşıl sökme ve ağartma gibi pek çok tekstil terbiye işleminde güçlü kimyasalların yerini almaktadır. Enzimlerin katı yüzeylere immobilize edilmeleri geri dönüşümlerini sağlamaktadır. Pamuklu kumaşların ağartılmasında enzimatik olarak üretilen peroksit kullanılmaktadır. Ticari Glukoz Oksidaz (GOx) değişik yüzeylere immobilize edilmiş ve GOx' in çapraz bağlı enzim çökeltileri hazırlanmıştır. Bunların glikoza olan aktiviteleri peroksit üretimleri bazında incelenmiştir. Bunun yanında, pamuklu kumaş üzerinde bulunan nişasta haşıl, amiloglukosidaz kullanılarak glikoza parçalanmıştır. Bu flotte, GOx ile peroksit üretiminde kullanılmıştır. Immobilize enzimlerin haşıl sökme flottesine karşı aktiviteleri ile pamuklu kumaşların üretilen peroksitle ağartma işlemleri sonrasındaki beyazlık değerleri incelenmiştir. Immobilize enzimle yapılan ağartmalarda ulaşılan beyazlık değerlerinin beyaz tekstiller için uygun olmadığı ancak boyanacak tekstiller için ulaşılan beyazlık değerlerinin yeterli olduğu belirlenmiştir. Haşıl sökme ve ağartma işlemlerinin tek banyoda birleştirilmesi ve enzimlerin geri dönüşümü çevreye yararlı bir alternatif oluşturmaktadır.

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ABBREVIATIONS

AATCC	Association for American Textile Chemists and Colorists
AG	Amyloglucosidase or glucoamylase
APTS	3-Aminopropoxytriethoxysilane
BSA	Bovine serum albumin
BTCA	Commercial peroxide stabilizer
CBD	Cellulose binding domain
CBM	Carbohydrate binding module
CBH	Cellobiohydrolase
CIE	Whiteness index unit
CLEA	Crosslinked enzyme aggregates
CLEC	Crosslinked enzyme crystals
CMC	Carboxymethyl cellulose
DNS	Dinitrosalicylic acid
G(-)	Native Gempil 4L
G(+)	Crosslinked Gempil 4L
Glu	Glutaraldehyde
GOx	Glucose oxidase
GPTS	3-Glycidoxypentyltrimethoxysilane
EG	Endoglucanase
IA(-)	Native IndiAge 2XL
IA(+)	Crosslinked IndiAge 2XL
IGOx	Immobilized Glucose oxidase
JA	Jeffamine
K_m	Substrate concentration where half maximal velocity of an enzymatic reaction is reached
LR	Liquor Ratio
NaOAc	Sodium acetate
NGOx	Native Glucose oxidase
PEG	Polyethylene glycol
RPM	Revolutions per minute

SDS-PAGE	Sodium doedecyl sulphate-polyacrylamide gel electrophoresis
SEM	Scanning Electron Microscopy
V_{\max}	Maximum velocity of the enzymatic reaction
WI	Whiteness Index according to Stensby equation

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CHAPTER 1

BACKGROUND

Enzymes are used extensively in the industrial processes. Nowadays extensive research has been done to replace conventional chemical processes and to improve the properties of the fabrics in textile industry. Enzymatic approach is found to be suitable for replacing those harsh chemical processes. Moreover, the building blocks of the fabrics are also substrates for many enzymes. Enzymatic applications are used to improve the properties of the fabrics as well as to get the desired properties. Enzymes are preferred in textile processes because of their specificity, speed, biodegradability, operational stability and vast application areas.

Enzymes are especially used in biofinishing and biopreparation of the textiles. These biopreparation and biofinishing processes change according to the fiber or fabric type to be processed. Enzymes are involved in desizing, bleaching, bioscouring, biopolishing and biostoning.

1.1 TEXTILE MATERIALS

1.1.1 Starch

Starch is one of the industrially important polymers. It is used widely in food industry, mainly for the production of glucose and it is also used in textile and paper industries for coating of the warps threading from the surface of the fabric and for

coating of the surface of the paper, respectively. Starch has been used as a sizing agent for more than hundred years in textile industry. Main sources of commercial starch are cereal grains such as corn, wheat, rice and roots such as potato.

It is a polymer of $\alpha(1\rightarrow4)$ and $\alpha(1\rightarrow6)$ linked glucose. $\alpha(1\rightarrow4)$ linkages form the linear polymer whereas $\alpha(1\rightarrow6)$ linkages introduce branch points to the starch polymer. As a result, starch is a branched glucose polymer. α -amylase only catalyzes the hydrolysis of $\alpha(1\rightarrow4)$ linkages however glucoamylase catalyzes both the hydrolysis of $\alpha(1\rightarrow4)$ and $\alpha(1\rightarrow6)$ linkages. It follows to reason that glucoamylases can hydrolyze starch into glucose much more efficiently than α -amylases. Starch is composed of two parts: i) amylose (Figure 1.1a)

ii) amylopectin (Figure 1.1b)

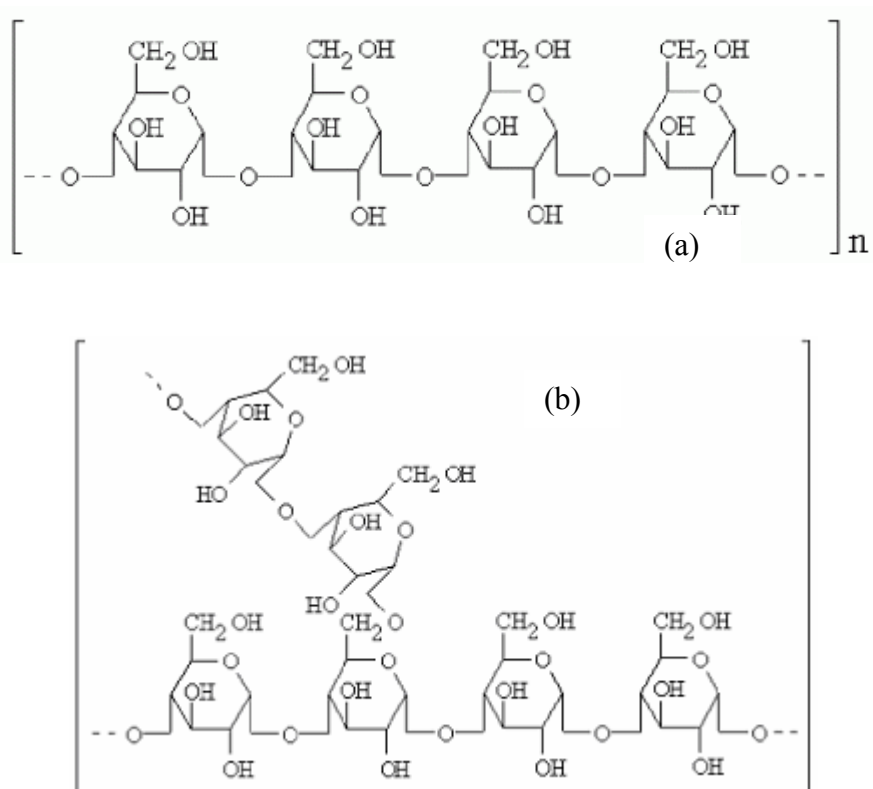


Figure 1.1: Structure of starch polymer: amylose(a), amylopectin(b) [32].

Amylose is a linear chain of glucose molecules having a molecular weight of 10^6 . Normal starch is made up of 25 % amylose. Amylose forms a right-handed helix due to its axial-equatorial link of $\alpha(1\rightarrow4)$. Amylose reacts with iodine to give a blue color.

On the other hand, amylopectin is a branched polymer of glucose molecules and it is one of the largest polymers on earth having a molecular weight of $\sim 10^7$ - $5 \cdot 10^8$. Common starch contains nearly 75 % amylopectin and contrary to amylose it does not show a helical structure because of the $\alpha(1 \rightarrow 6)$ linkages that disrupts H-bonding pattern in the helical structure. Starch polymer exhibits a highly ordered structure and crystalline regions comprises ~ 35 - 40 % of the total structure. Amylose and amylopectin are subject to hydrolysis by alpha amylases and glucoamylases to different extents. Figure 1.2 indicates the enzymatic action of starch degrading enzymes, alpha-amylase and glucoamylase on amylose and amylopectin.

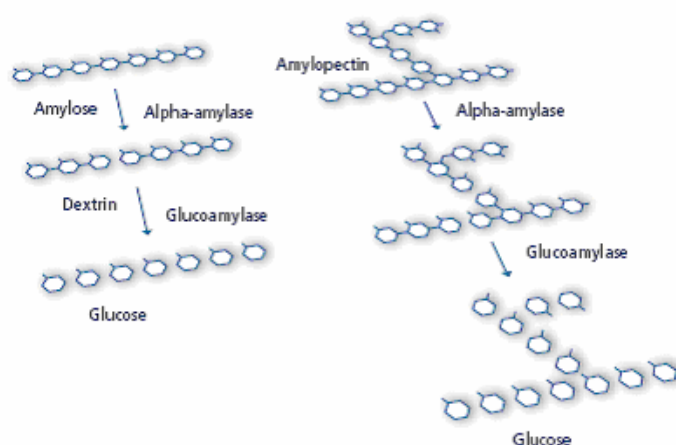


Figure 1.2: Enzymatic action of alpha-amylase and glucoamylase on amylose and amylopectin [32].

1.1.2 Cotton

Cotton is the most important textile fiber source. Raw cotton comprises nearly 36 % and 56 % of all world textile fiber production in 2000 (Heikinheimo, 2002) [25] and in 2004 [28]. It is a natural cellulosic fiber. Cotton use is very widespread owing to its desirable fiber properties with respect to other fibers. It has a comfortable soft hand, good absorbency, strength, color retention and it prints well, drapes well and it has many end uses as apparel and in home fashion. On the other hand, it is not resistant to abrasion or wrinkling.

Cotton plant belongs to the genus *Gossypium*, and cotton is the seed hairs of the cotton plant. It has a fibrillar structure and composed of concentric layers. It is composed of a primary cell wall on the outermost layer, a secondary cell wall in the middle and a lumen inside (Figure 1.3).

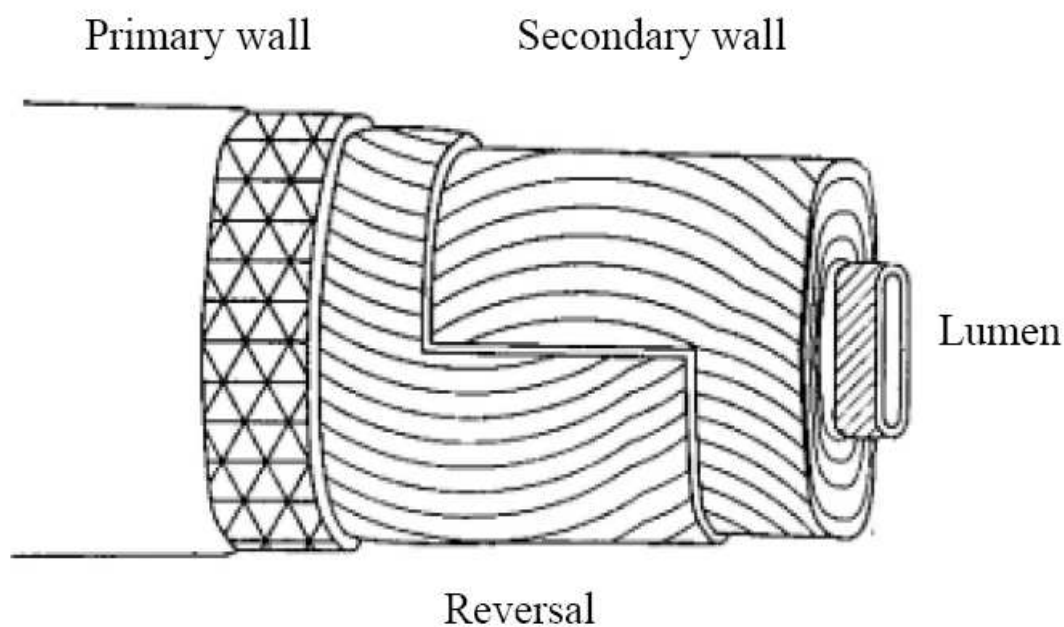


Figure 1.3: Illustration of cotton structure (cited in Heikinheimo, 2002, Nevel, 1995) [25].

The cuticle layer consisting of wax, lipid and pectins is on the fiber and can be separated from the fiber. The primary cell wall is composed of crystalline fibrils made up of cellulose and also contains some impurities such as lipids, waxes, proteins, pectins etc. The secondary cell wall comprising most of the mature fabric and also the cellulose part, has three sublayers. These layers are composed of cellulose fibrils arranged with a spiral winding around the fiber axis. The lumen present in the inner part contains the remaining cell contents. The cotton fiber has a bean shaped cross section and it seems round when it swells upon moisture absorption. On the overall, raw cotton is composed of 80-90 % cellulose, 6-8 % water, 0.5-1 % waxes and fats, 0-1.5 % proteins, 4-6 % hemicelluloses and pectins and 1-1.8 % ash [25].

Cotton structure is composed of mainly crystalline fibrils differing in complexity and length. The crystalline regions are connected by amorphous regions that have more

loose structures than crystalline regions. Crystalline regions comprises nearly the two thirds of the total structure.

1.1.3 Cellulose and Viscose

Cellulose is an unbranched polymer of β -1,4-linked glucose molecules. Plants are the only producers of cellulose. It is the most abundant polymer on earth. The glucose units forming the cellulose chain are in six membered pyranose ring. There forms an acetal linkage between the C1 of one pyranose ring and C4 of the next pyranose ring. A single oxygen atom joins the two pyranose rings. In the formation of an acetal, one molecule of water is lost when an alcohol and a hemiacetal reacts. That is why the glucose units forming the cellulose polymer are also called anhydroglucose units.

The spatial arrangement of the acetal linkages are very important in determining the characteristics of the cellulose molecule. With the formation of pyranose ring, there exists two possibilities for the configuration. The hydroxyl group present on C4 can approach the C1 hydroxyl group from both sides. This results in two different stereochemistries. If the C1 hydroxyl group is on the same side with C6 hydroxyl group, the configuration is called the α , if they are on the opposite sides the configuration is called the β . Cellulose is known to be in β configuration and is a poly[β -1,4-D-anhydroglucopyranose]. In β configuration all the functional groups (hydroxyls) are in equatorial positions which means they protrude laterally from the extended molecule. These protruding hydroxyl groups are readily available for hydrogen bonding (interchain and intrachain hydrogen bonding are observed). Moreover, inter and intrachain H-bonding and Van der Waals interactions force the cellulose chains into a parallel alignment and finally to an ordered crystalline structure. This property allows the chain of cellulose to extend in a straight line and makes cellulose a good fiber forming polymer by giving tensile strength along the fiber axis. Hydrogen bonding causes the formation of highly ordered crystal structure. This highly ordered crystalline regions are thought to be intruded with less ordered amorphous regions (Zhang et al, 2004) [64]. The interchain hydrogen bonds in the crystalline regions gives the fibers their strength and insolubility. In the less ordered regions the cellulose chains are more

loose and further apart as a result. This enables the hydroxyl groups to form hydrogen bonds for example with water molecules and causes these regions to absorb water. On the other hand, amylose has the α configuration, C1 oxygens are in α configuration. This causes the formation of the linkages between the adjacent glucopyranose residues to be in axial positions and this forces the amylose chain to assume a helical structure maintained by interchain hydrogen bonds. Since helical structure is not proper for fiber formation, starch is not a suitable fiber-forming molecule.

Figure 1.4 indicates the repeating unit of a cellulose molecule. The repeating unit of cellulose is the anhydrocellobiose (Zhang et al., 2004) [64]. Cellobiose is formed from two identical but 180° rotated anhydroglucose units. This introduces the symmetry to the cellulose molecule since there are equal numbers of hydroxyl groups on each side of the molecule.

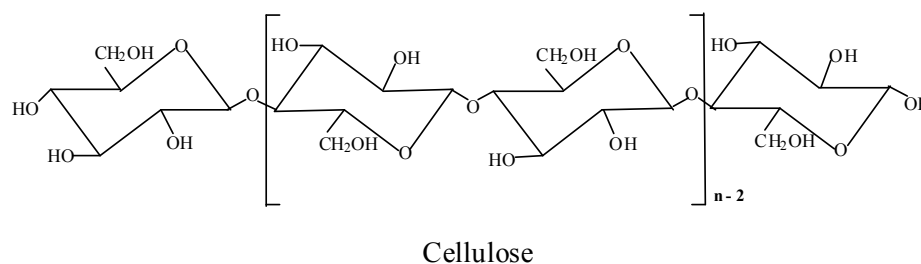


Figure 1.4: Repeating unit of a cellulose molecule.

Native cellulose is known as Cellulose I and it can be converted into other crystalline forms (Cellulose II-IV) with various treatments mentioned in the literature (cited in Zhang et al., 2004, Colom et al., 2002, Shin et al., 2000) [65, 13, 57].

Glucopyranose rings of cellulose are in the form of stacked sheets, on top of each other forming a three-dimensional structure. Because of this arrangement the surface of the cellulose is composed of different “faces” such as 110, 1-10 and 020. Figure 1.5 shows the surface structure of the cellulose I crystal with its repeating anhydroglucopyranose rings (Zhang et al., 2004) [64]. All six carbons of the glucopyranose rings and acetal link is in 110 surface which is in the ab plane. On the other hand, the edges of the rings are in the 1-10 face, in the ac plane [64]. The cellulose molecule interacts with water molecules in an aqueous environment and with cellulases

via these faces. Cellulose crystals have two nonidentical surfaces, 110 and 1-10 and Henrisatte et al. (1988) (cited in Reinikaen et al., 1995) [51] indicated with electron microscopy data from algal cellulose crystals that CBHI (Cellobiohydrolase I) adsorbs preferentially to the 110 surface.

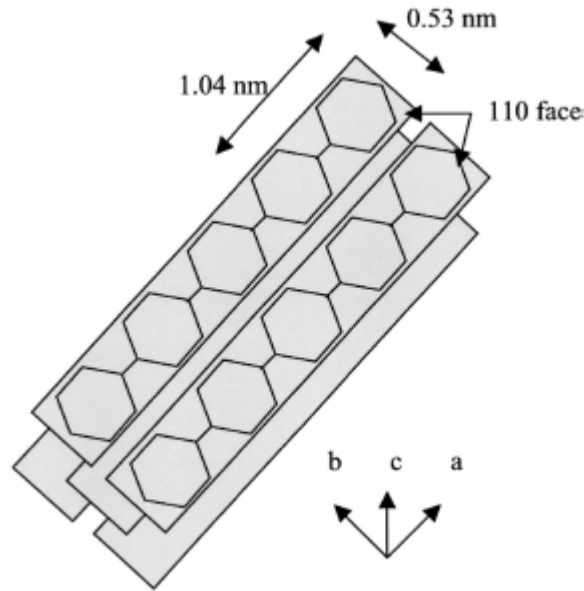


Figure 1.5: Structure of cellulose I crystal with its anhydroglucopyranose units. 110 face is in the ab plane [64].

Researchers have been working on the production of artificial silk from cellulose by forming cellulose derivatives for years. These researches introduced two commonly used routes for the production of fibers: acetate and xanthate esters. Cellulose acetate is a cellulose derivative and soluble in solvents such as acetone and can be spun into fibers. When cellulose is exposed to strong alkali and then treated with C_2S , xanthate esters of cellulose are formed. Cellulose xanthate is soluble in alkali (aq.) and from this solution filaments and films can be formed. Cellulose xanthate process is the basis of viscose rayon production [edt. Brown, R.M.] [17].

Viscose is a regenerated cellulose fiber and it has a high tenacity and high extensibility. It is manufactured from cotton linters or from cellulose obtained from wood pulp. Viscose process requires many steps. Viscose fabrics are less strong than cotton fabrics. Viscose has a very low mechanical strength especially when it is wet.

Viscose is composed of both amorphous and crystalline cellulose with different ratios. The viscose fiber consists of a core surrounded by the mantle, of which crystalline and amorphous cellulose content differs (Figure 1.6). The crystalline regions in the mantle are smaller and distributed homogeneously throughout the fiber and the core region contains a disordered network formed from bigger crystallites separated by big amorphous regions. The outer region is more ordered and rigid than the core region and accounts for the most of the tensile strength.



Figure 1.6: Cross-section of a viscose fiber.

It is known that amorphous cellulose is more prone to attack by cellulases than crystalline cellulose. Crystalline cellulose is more rigid and gives tensile strength along the fiber axis whereas amorphous cellulose is mainly responsible for the flexibility. The loss of tensile strength is most probably due to the loss of highly ordered crystalline structure by the action of cellulases.

1.2 TEXTILE PROCESSES

1.2.1 Desizing

Desizing refers to the removal of the size on the fabric. Usually cotton fibers and its blends are coated with starch or starch based material in order to give strength to the fibers and to prevent fiber damage during the process of weaving due to the harsh physical conditions of the process. These harsh physical conditions built a mechanical stress on the warps threading from the surface of the fabric. There are different types of sizes. Starch and starch based materials are most commonly used as sizes however other polymeric materials such as polyvinyl alcohol (PVA), carboxymethyl cellulose (CMC) and triglyceride can also be used. Starch is preferred (75 %) because of its availability, biodegradability and lower price. The removal of the size from the surface of the fabric after the weaving process is important since the fabric would be subjected to wet processing such as bleaching and dyeing and wet processing requires the fabric to be uniform. Conventional desizing includes the application of oxidative chemicals onto the fabric and starch degrading enzymes such as alpha-amylases and glucoamylases are replacing the oxidative chemicals nowadays owing to the fact that they are highly efficient and they specifically attack starch, not the fibers and the fabric. Moreover thermostable starch degrading enzymes are commercially available to increase the desizing yield.

1.2.1.1 Desizing Process

Desizing process with enzymes is composed of three stages. These are impregnation, incubation and afterwash. Before all these steps a prewashing step could be added for the removal of the water-soluble additives on the fabric and for easy binding of the enzyme onto the starch granules. In the prewashing step starch was gelatinized by wetting in hot water (80-90 °C). Then at the next step, alpha-amylase enzyme (or glucoamylase) is added with a suitable buffer solution at optimum pH and temperature. This process is followed by

a longer incubation step (2-16 hours). The process time depends on the properties of the enzyme used in the process. The soluble dextrin molecules that are produced as a result of the enzymatic action are removed via an afterwashing step and the pH of the medium is neutralized for the next scouring and bleaching steps. Desizing efficiency is predicted by using the iodine color test. Starch reacts with iodine in water giving a deep blue-black color.

There are three types of processes used in desizing; batch, semi-continuous and continuous and there are different methods used for each type of process. For example, jigger method is a kind of batch process. In this method fabric from one roll is processed in a bath and rewound on another roll at the end of the bath. It is relatively simple. In modern continuous high speed processes because of the discovery of highly thermostable and reactive amylases the process times are in the scale of seconds. Desizing performed on pad rolls is a continuous process in terms of the passage of the fabric with the exception of the use of thermo-labile amylases since their utilization requires longer incubation times. Emergence of thermostable amylases allow the desizing process to be performed in steam chambers at $\sim 100^\circ\text{C}$. This makes the process fully continuous. Figure 1.7 indicates this process.



Figure 1.7: A pad-roll process showing each desizing process [49].

1.2.1.2 Enzymatic Desizing

Although hydrolysis of starch by amylolytic enzymes has been known for more than a hundred years, use of amylases for desizing of textiles have been introduced in the last decade. Feitkenhauer et al. (2003) introduced microbial desizing as a new bioprocess. This process uses the acidifying culture of a two-phase anaerobic digestion plant for the removal and partial degradation of the sizing agent and this process is

proven to be efficient in terms of starch solubilization. They found an acceptable decrease in degree of polymerization of starch after 1h incubation [18]. Use of glucoamylase instead of amylases in the desizing process was found to be more effective in terms of glucose production (Buschle-diller et al., 2001) [9].

1.2.2 (Bio)bleaching

The purpose of bleaching process is to reach proper whiteness values for the fabrics, yarns or fibers for use in further processes such as dyeing. High whiteness values are needed for non-dyed and pastel shaded fabrics whereas lower whiteness values are sufficient for dark coloured fabrics (Lange et al., 2001) [39]. Bleaching agents such as hydrogen peroxide, sodium chlorite and sodium hypochlorite are used in conventional bleaching processes. Addition of many chemicals such as stabilizers, surfactants, chelators/sequestering agents to the bleaching baths creates an environmentally unfriendly situation. Hydrogen peroxide bleaching is preferred over other bleaching methods and nowadays enzymatically produced hydrogen peroxide is under trial.

1.2.2.1 H₂O₂ bleaching

H₂O₂ is a very suitable bleaching agent for natural fibers derived from cellulose such as cotton, linen and bast fibers. Conventionally, cotton is treated with H₂O₂ solution by the addition of a stabilizing agent, namely sodium silicate. Bleaching of cotton is performed at 80-95 °C in alkaline bleaching baths (~pH 11) (The bleaching time is generally between 2 and 5 hours) but with the steam baths this process could be performed at temperatures as high as 130°C and within very much shorter process times (7-20 min). In cold bleaching processes, this takes much longer time (18-24 hours).

Various processing stages (loose stock, yarn, woven fabrics and knitted fabrics) and various types of mechanical bleaching equipment (jig, winch etc.) are used for

bleaching according to the type of the stage. For example since the properties of the yarn is different from the properties of the woven fabric different types of equipments are used to fulfill the needs. As mentioned above the purpose of bleaching is to reach a permanent brightness without harming, weakening the fibers that is without changing the degree of polymerisation of the fibers.

There are many advantages of use of peroxide as a bleaching agent. First, it provides the highest bleaching effect with reasonable costs due to short term bleaching processes. Second, peroxide bleaching does not target the fibers so the quality of the fiber is not reduced. Third, cotton bleaching is a one-step process whereas use of other processes involves some additional steps. Moreover, residual peroxide is still present in bleaching baths after the bleaching process and this helps in the degradation of the organic impurities in the effluent and thereby decreasing the chemical oxygen demand (COD).

The bleaching bath is composed of hydrogen peroxide (35% or 50% by weight) as the bleaching agent, a peroxide activator and stabilisers. Generally, the peroxide bleaching bath is activated by alkali. Cotton is bleached in caustic soda solutions however the type of alkali to be used and the pH depends on the type of the fabric and pretreatment. For example to avoid cottonizing weaker alkaline or soda alkaline bleaching baths are used for bleaching of linen and linen-like fibers. Moreover, since regenerated cellulose fibres are more sensitive to alkaline only weak alkaline baths are used for bleaching [27].

Alkaline pH is important for the activation of the peroxide but one major drawback is that at high pH, peroxide is destabilized and it decomposes rapidly. This causes a reduction in the whiteness values. To prevent decomposition of the bath, stabilisers must be added. Sodium silicate or organic stabilizers or their combination could be used in caustic alkaline bleaching baths. Interestingly, the use of tetrasodium pyrophosphate provides peroxide activation as well as peroxide stabilisation. Additionally some wetting agents/surfactants and detergents are also used with dry and untreated fibres. These agents allow to obtain a quick and through impregnation of the textile to be bleached by the liquor. The chemicals that help to prevent the

precipitation of carbonates and silicates from hard water are also suitable for use in the bleaching process.

The type, quality, origin of the textile, the liquor ratio, the required whiteness values determine the percent consumption of hydrogen peroxide. The term "liquor ratio" refers to the relationship of the goods being bleached to the volume of bleaching solution.

The removal of hydrogen peroxide after bleaching is very important since it affects the efficiency of afterdyeing processes. This problem was originally solved by rinsing with water repeatedly. The emergence of the enzyme technology allowed us to use catalase enzyme which decomposes peroxide rapidly to water and oxygen. Catalase is a peroxidase enzyme and it catalyzes the hydrolysis of peroxide into water and oxygen. This enzyme proved its success in efficient removal of even trace amounts of peroxide present on the fabric or yarn. This brings the advantage of lowering the costs by saving from water, time and energy. Moreover, it is environmentally more friendly. Total removal of peroxide is important because the dyes to be used in the next step are peroxide sensitive dyes and it is known that 10ppm of residual H_2O_2 or more causes dyeing problems, although it depends on a type of dye [29]. The enzyme's specificity is only towards peroxide so that it does not interfere with the other stages of the process such as dyeing.

1.2.2.2 Enzymatic bleaching

Enzymatic bleaching has emerged as an alternative to conventional bleaching to reduce the environmental pollution by reducing biological oxygen demands (BOD). First studies on enzymatic bleaching has been concentrated on the bleaching of pulp for paper and members of oxidoreductase enzyme family has been used. These enzymes are capable of increasing the oxidative power of oxygen or hydrogen peroxide.

Few researchers have been working on the optimization of bleaching process with enzymatically produced H_2O_2 (Buschle-diller et al, 2001, Tzanov et al, 2002) [9,59].

Research is mostly concentrated on improving the whiteness levels of the fabrics, yarns or fibers.

1.2.1.3 Cotton Biopreparation

Cotton biopreparation includes bioscouring, biobleaching and mercerization. It is a process in development. Biopreparation targets the impurities present in cotton and researchers have shown that these impurities could be removed by the application of specific enzymes. For example impurities like lipids are removed by the action of lipid hydrolyzing enzymes, lipases and proteins by proteinases, pectins by pectinases etc. Main goal of these enzymatic applications includes the removal of those impurities for increasing the wettability and whiteness levels of the fabric or yarn.

Buschle-diller et al. (2001) was the first to introduce a closed loop system for the biopreparation of cotton. Their closed loop system includes desizing, scouring and bleaching at the same bath one after another. This system uses the glucose produced by desizing with a glucoamylase enzyme. Glucose oxidase (GOx) is used to produce peroxide from glucose via glucose oxidase and bioscouring was performed with pectinases. They reached to whiteness indexes of 63-64 according to Ganz equation with the three-step combined desizing-bleaching and scouring and the whiteness of the conventionally scoured and bleached fabric was 74 [9].

In 2002, Tzanov et al.. immobilized GOx on glass and alumina support and determined the efficiencies and stabilities of each support for H₂O₂ production and for repeatable use. They found that GOx immobilized on glass support was more active than the GOx immobilized on alumina support. On the other hand, the operational stability of the alumina support was found to be better than glass support. Whiteness levels produced by immobilized GOx was comparable to the whiteness levels produced by the free enzyme and commercial H₂O₂. CIE standard was used to evaluate the whiteness of the fabrics. According to CIE immobilized enzyme treated fabric whiteness index was 74, free enzyme treated fabric whiteness was 73.5 and whiteness of the fabric bleached by commercial H₂O₂ was 74.5 [58].

1.2.3 Biopolishing

Biopolishing also known as biofinishing refers to the removal of the cellulose fibrils and microfibrils protruding from the surface of the fabric or fibers by the action of cellulases. These fibrils and microfibrils are termed as fuzz. These loose microfibrils and fibrils tend to agglomerate on the fabric surface. These loose agglomerations are called pills. Pills are formed during fabric processing in the production plant, washing and/or wearing. The mechanical action provided by the friction of the fabrics during wearing causes pill formation. There is an increasing demand on the use of cellulases in the textile industry for the removal of pills and fuzz formed on the surface of the fabric. Enzymatic treatment provides fabrics with

- better surface properties and look
- improved hand properties
- improved drapeability (ability to hang or stretch out loosely)
- increased brightness
- reduced pilling and pilling tendency
- increased softness compared to the conventional softeners

Biopolishing involves the enzymatic treatment of the cellulosic fabrics such as cotton, linen, rayon and Lenzing's Lyocell and viscose with cellulases that eventually leads to the weakening of the fibers protruding from the surface of the fabric and the removal of the weakened fibers with mechanical action. The tendency for the formation of pills during wearing and washing is minimized since the protruding fibrils are removed by biopolishing. The biopolishing process was patented in 1993 by Videbaek and Andersen [59] and it is mainly designed to improve fabric quality.

Since biopolishing is an enzymatic process it can be carried out during the wet processing stages. It is mostly performed after bleaching before dyeing. After bleaching, the fabric becomes cleaner and more hydrophilic. So it becomes more prone to attack by cellulases. Biopolishing is not performed after dyeing since there is risk of color fading

and the chemical content of the dyes can reduce the performance of the enzymes by interfering with them. Direct and reactive dyes have been known to have an inhibitory effect on cellulases.

Biopolishing is mostly performed in machines such as a jet-dryer or winch. Enzyme dosage is a very important parameter for having the desired effect. The dosage was determined as a percentage of the garment weight. Usually, 0.5-6 % enzyme over fabric weight is used by the manufacturers. Process parameters such as pH, temperature and duration is determined according to the properties of the cellulase enzyme to be used. Generally the process is performed at pH 4.5-5.5, temperature between 40-55 °C for 30-60 minutes and the enzyme is inactivated usually by increasing the temperature above 80 °C or pH above 10. Soda ash is used for the pH adjustments.

Many aspects of cotton biopolishing with cellulases were studied. For example, Miettinen-Oinonen et al. (2005) [44], developed different cellulase formulations (CBH I, CBH I and II, EG II, EG I and II enriched and wild type) by genetic engineering and applied these on the biofinishing of cotton fabrics. They found that EG II enriched and EG enriched cellulase formulations improved the surface appearance more than CBH I, CBH II and CBH enriched cellulase formulations. All the pilling values were better than the wild type and CBH II was found to be the most effective throughout all CBHs. The pilling values for EG II enriched cellulase formulation was 4.3 and for CBH I enriched cellulase and wild-type cellulase pilling values were 2.3 where a pilling value of 5 indicates no pills and 1 indicates intense pilling.

Although there are many studies on the biopolishing of cotton fabrics, there are a few on the biopolishing of the regenerated cellulose fabrics such as Lyocell and viscose. Use of cellulases in biopolishing of viscose was studied by Ciechańska et al.(2002) [12]. Different formulations of cellulases (EG II, CBH I and total cellulase enriched with EG II) and a commercial cellulase (Econase CE, Rohm Enzymes Inc.) from *T. reesei* were applied on two types of viscose woven fabrics. The microscopic properties of the fabrics and residual fibers were analyzed, but the pilling values or pilling tendencies were not evaluated. It was found that use of the commercial enzyme removed most of the microfibrils and fuzz protruding from the surface of the viscose woven fabric A

(Figure 1.8a). But the purified components did not improve the surface of the viscose woven fabric B (Figure 1.8b).

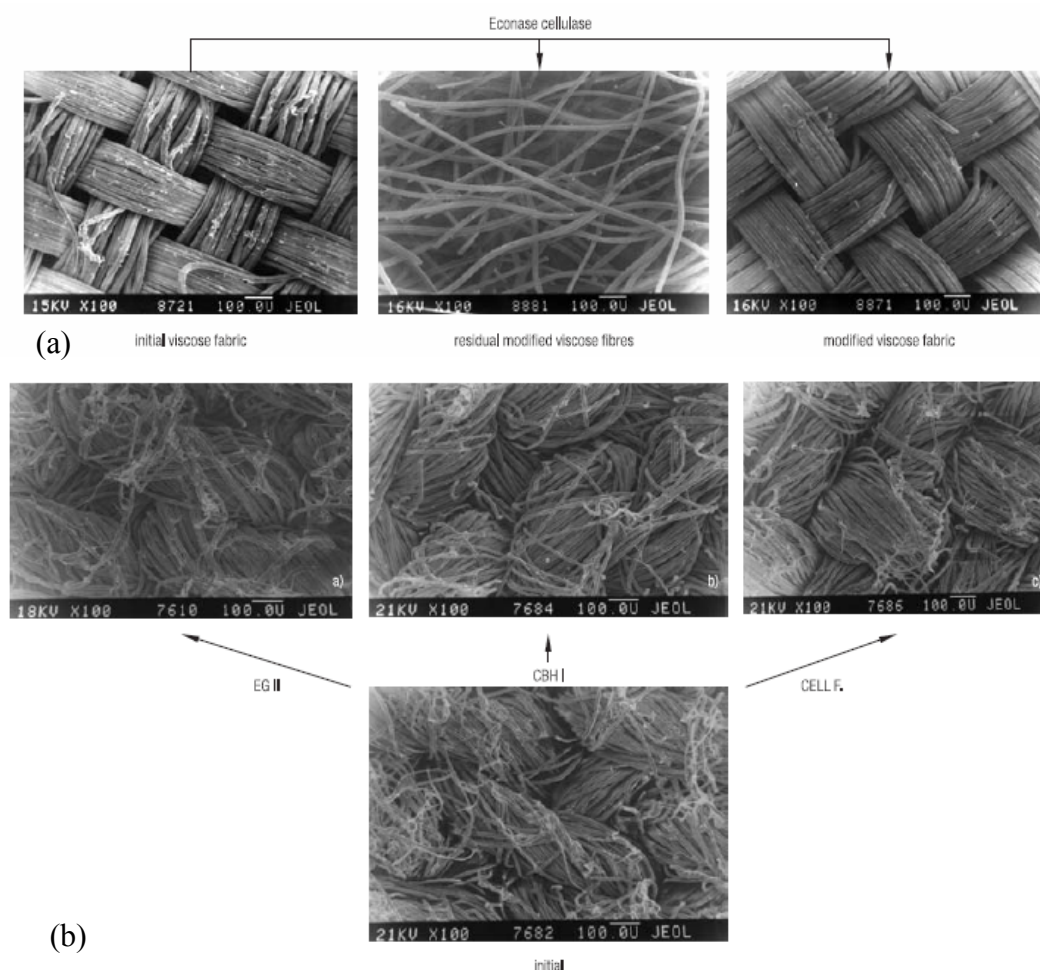


Figure 1.8: (a) Viscose woven fabric A untreated, treated with Econase CE. (b) Viscose woven fabric B untreated, treated with EG II, CBH I and EG II enriched cellulase CELL F [12].

According to our knowledge, there are no studies on the biopolishing of knitted viscose fabrics and their pilling values upon enzymatic treatment. Liu et al. (2000) (cited in Heikiheimo, 2002) [25], analyzed the effects of different commercial and experimental cellulase preparations (commercial multicomponent acid cellulase and monocomponent acidic endoglucanase, experimental EG enriched cellulase) on cotton interlock (type of a stretchable fabric) knitted fabric. According to their results and interpretation, cellulases were found to have different selectivities when their ratios of pilling to bursting strength, their sensitivities to liquor ratio and mechanical agitation created by the equipment, fiber types were considered. Kumar et al.(1999) suggested

that EG enriched cellulases had some advantageous properties such as improved hand compared to total cellulase preparations (cited in Heikinheimo, L., 2002) [25].

Effects of many surfactants such as Tween 20 and Tween 80 on cellulase activity were analyzed by Mizutani et al. (2002) [46], Ooshima et al. (1986) [50], Kaar et al. (1998) [35], Castanon et al. (1981) [10]. It was found that long incubation times more than 4 hours are needed for the effect of Tween 20 [46, 50], more than 10 hours are needed for Tween 80 [10].

One of the problems encountered during biopolishing is the loss of fiber or fabric strength as a result of the aggressive action of the enzymes. This problem is predominantly seen in the biofinishing of lyocell and viscose fabrics. These problems are solved using different formulations of cellulases (Kumar et al., 1997) [37]. Viscose fabrics' tensile strength is known to be lowered when it is wet. This poses an important problem since most of the textile processes are wet processes. Moreover, aggressive cellulases are used in most of the processes. There are commercial cellulase preparations suitable for lyocell biofinishing and most of them are also suggested for biofinishing of viscose fabrics. But to our knowledge there are no specific commercial cellulases for viscose and the ones that are used for lyocell, cotton are insufficient for the removal of the pills on viscose (especially, viscose knitted fabric).

1.2.4 Biostoning

Stone-washing is applied to the denim fabric. Most denim fabrics are subjected to stoning in order to be given a worn look and aesthetic appearance. Some of the dye present on the surface of blue colored denim fabric is removed by the abrasive action of pumice stones in the traditional stoning process. Nowadays, cellulases are replacing the use of pumice for stoning since they are less aggressive and the use of cellulases for stoning of the denim is called biostoning. The use of cellulases in place of pumice stone is proven to be advantageous since the target of cellulases is mainly the indigo dye present on the surface of the fabric. The action of cellulases causes the loosening of the indigo dye on the denim and which in turn accelerates the abrasion. Also small

quantities of the enzyme can have the effect of several kilograms of the pumice stone. This replacement also minimizes the negative effects of the pumice stones on the fabric as well as the washing-machines. Moreover, use of enzymes is an environmentally friendly alternative [49].

Cellulase action is mainly restricted to the fibrils protruding from the surface of the yarn that make up the denim. Indigo dye adheres to the surface of the yarn and cellulases acts on the fibrils on the surface of the yarn. As a result of the cellulase action the fibrils are hydrolyzed without giving any damage to the interior part of the cotton fiber (Figure 1.8). So, the mechanical strength of the cotton fibers is not reduced. Since the indigo dye present on the surface of the fibrils is partly removed by the hydrolysis, light colored areas form on the denim surface. Neutral and acid cellulases are used for the biostoning of the denim.

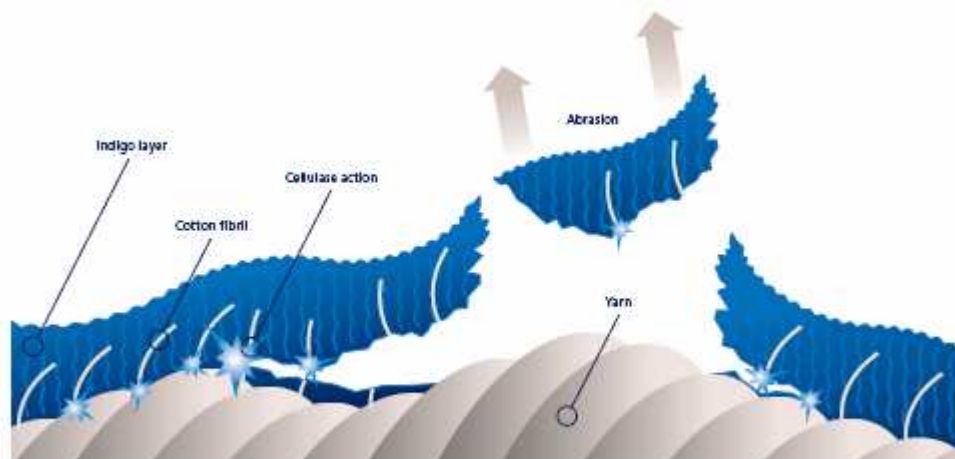


Figure 1.8: Stone washing effect of cellulases on denim fabric [49].

Backstaining, that is the redeposition of the indigo dye on the denim is an important problem. This problem could be solved by the use of neutral cellulases. Backstaining is minimal at neutral pH values and higher at lower pH values.

1.2.5 Bioscouring

Cotton fabric is processed before dyeing. One important step prior to dyeing is the scouring process. Scouring targets the noncellulosic impurities present in the cotton fibers or fabrics. These impurities consist of pectins, waxes, lipids and some proteins etc. For the removal of these impurities cotton is treated with NaOH conventionally. Use of conventional method increases the environmental pollution by increasing Chemical and Biological Oxygen Demands and salt content. Nowadays different enzymes are used in bioscouring processes. Pectinases are used to remove pectins, lipases to remove lipids and proteinases for the removal of proteins. The purpose of scouring is to prepare the cotton fabrics or yarns for dyeing processes by giving a high and even wettability to the fabric [49].

1.3 TEXTILE ENZYMES

1.3.1 Desizing Enzymes

Alpha-amylases and amyloglucosidases are the enzymes that are used in the degradation of starch and starch like polysaccharides. They are also used in the desizing process to hydrolyze starch. Both alpha-amylases and amyloglucosidases belong to the hydrolase family and glycosyl hydrolase subfamily of enzymes.

Amylases were first discovered in 19th century. The first starch degrading enzyme was discovered by Kirchhoff [49]. Later some reports on malt and digestive amylases appeared and surprisingly in 1930, the classification of these enzymes as alpha and beta in malt was suggested by Ohlsson. This classification was based on the anomeric type of the sugar produced as a result of the enzyme reaction. Amylases can further be categorized into two. Endoamylases perform random hydrolysis of glucosidic bonds in the interior of the starch molecule resulting in long linear and branched oligosaccharides differing in chain lengths. On the other hand, exoamylases as the name implies performs hydrolysis from the nonreducing ends, successively, producing short

chains of oligosaccharides. For the complete hydrolysis of starch, the combined action of these enzymes are required (Robert et al., 2003) [52].

Alpha-amylases (1,4- α -D-glucan glucanohydrolase; EC 3.2.1.1) are monomeric enzymes (Figure 1.9) [30]. They are synthesized by a wide range of living organism from microorganisms to plants and animals. They catalyse the endohydrolysis of α -D-(1,4)-glucosidic linkages present in starch (in amylase and amylopectin), glycogen and similar polysaccharides into maltodextrins and glucose.



Figure 1.9: Three-dimensional structure of *Aspergillus niger* acidic alpha-amylase (2AAA) [30].

Alpha-amylase is a metalloenzyme and it contains at least one calcium ion. In many organisms one Ca^{2+} is sufficient for the stabilization of the enzyme. The amount of Ca^{2+} bound to the enzyme varies between one to ten. For example Crystalline Taka-amylase A (TAA) has ten calcium ions and only one of them is tightly bound to the enzyme molecule (Gupta et al., 2003) [21]. The alpha-amylase produced by barley has at least three Ca^{2+} . Barley alpha-amylase isozyme AMY1 has four bound Ca^{2+} (Figure 1.10).

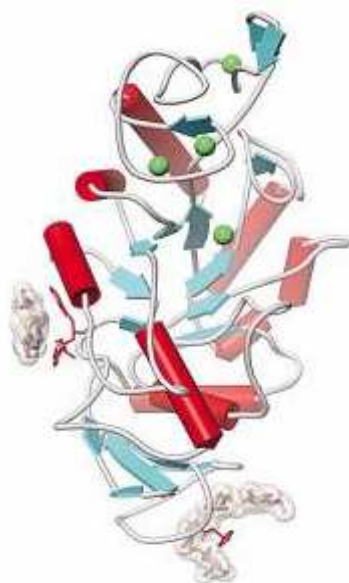


Figure 1.10: Overall structure of barley alpha amylase isozyme 1, AMY1 in complex with the substrate analog methyl 4^I,4^{II},4^{III}-trithiomaltotetraoside, Thio-DP4. The bound four calcium ions, one occupying the active site were shown as green spheres. The two tryptophan residues (Trp278 and Trp27) are highlighted in red to indicate the starch granule binding surface site. The bent structures at the bottom of the figure shows the substrate analog thio-DP4 [21].

The alpha-amylases of various organisms exhibit different enzymatic and physicochemical properties, but in general they have high affinity for starch. They also display specificity towards amylose, amylopectin, cyclodextrin, glycogen and maltotriose, but to a lesser extent.

Alpha-amylases exhibit pH and stability over a very wide range. Alpha-amylases from different origins have pH optima of 2 to 12.

Glucoamylases or amyloglucosidases (EC 3.2.1.3) (α -1,4-glucan-glucano hydrolase) are the enzymes that catalyze the hydrolysis of both $\alpha(1\rightarrow4)$ and $\alpha(1\rightarrow6)$ glucosidic bonds of starch thereby producing β -D-glucose as an end product. Because of their capability of hydrolysis of $\alpha(1\rightarrow6)$ linkages, glucoamylase can hydrolyse starch into glucose completely and much more effectively than alpha-amylases. Mechanism of action of amyloglucosidase enables the enzyme to hydrolyze the linkages at the branch points and as a result, more nonreducing ends are produced (Silva et al., 2004) [57]. The

activity of the glucoamylase towards the α -(1 \rightarrow 6) linkage is very small with respect to the activity towards α -(1 \rightarrow 4) linkages.

Glucoamylases belong to the glycoside hydrolase family of hydrolase enzymes and primary structures of many of the glucoamylases from filamentous fungi, yeast and archaea have been unravelled however only a few three-dimensional structure belonging to glucoamylases from different species were discovered. According to Protein Databank data, only crystal structures of *Thermoanaerobacterium thermosaccharolyticum* (Aleshin et al., 2003 [1]) and *Aspergillus awamori* var. X100 glucoamylases are fully resolved.

Being the main producer of many industrially important enzymes, *Aspergillus niger* also produces amyloglucosidase and this enzyme has been used for industrial purposes for decades. Preliminary structural studies have been performed on this enzyme and these studies revealed the structure of *A. niger* amyloglucosidase based on the structure of *Aspergillus awamori* var. X100. These two enzymes from two different species share 94 % sequence identity and as a result the two enzymes have most of their structural properties in common. Crystal structure of the starch binding domain of glucoamylase of *A. niger* in its free form and with its bound substrate has been found by NMR spectroscopy. Figure 1.11 indicates the crystal structure of the starch binding domain of *A. niger* glucoamylase with its bound substrate, L-cyclodextrin. Cyclodextrin is a molecule that mimicks starch. The molecular weight of the amyloglucosidase from certain varieties of *Aspergillus niger* was predicted to be 97 kDa.

Moreover, glucoamylases have one starch binding domain and one catalytic domain associated with a linker domain. Unfortunately, the crystal structure of all three domains together is not resolved yet. Studies with Scanning Tunnel Microscope are concentrated on revealing the relative positions of these domains (Sauer et al., 2000) [54].

It was shown that starch binding domain of glucoamylase was required for starch degradation. The G2 form of the enzyme having amino acids from 1-512 is devoid of the starch binding domain and it has been shown that the enzymes activity for raw

starch was reduced in the G2 form. Moreover, it has been recently shown that isolated starch binding domain acting on starch granules together with G2 indicated a synergistic effect during the hydrolysis. This suggests that starch binding domain facilitates the hydrolysis by the catalytic domain by binding onto starch and disrupting its compact structure. The starch binding domain contains two distinct sites for starch binding and it can also accommodate the starch analog L-cyclodextrin.

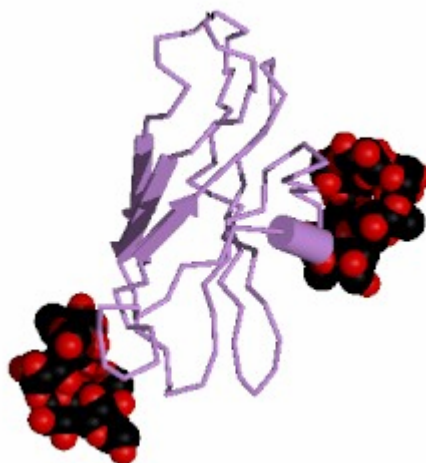


Figure 1.11: Three dimensional structure of granular starch-binding domain complex of amyloglucosidase with L-cyclodextrin [31].

General scheme for the hydrolysis of glucosidic linkages follows the general acid-base catalysis. Mutational analysis of *A. niger* glucoamylases revealed Glu179 (proton donor) as the general acid catalyst and Glu400 as the catalytic base. The general mechanism involves proton transfer from Glu179 to the glycosidic oxygen of the scissile bond, formation of an oxocarbenium ion and a nucleophilic attack of water assisted by Glu400 (Figure 1.12). Moreover, hydrolysis occurs with the inversion of the anomeric configuration with a single displacement mechanism. The gap between these two catalytic acids is found to be 9.2 Å. This is the typical property of inverting glycoside hydrolases. On the contrary, the distance between catalytic acids is 4.8-5.5 Å in retaining glycoside hydrolases and this causes the hydrolysis to occur by a double displacement mechanism involving a covalent intermediate (McCarter et al., 1994) [34].

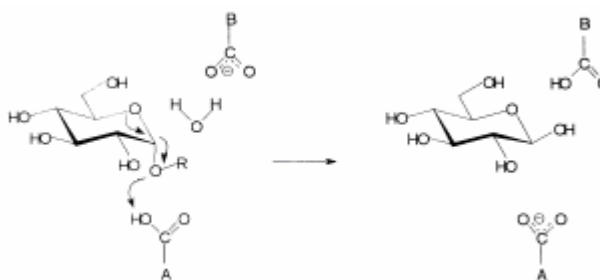


Figure 1.12: Widely accepted catalytic mechanism of glucoamylases indicating the action of Glu400 (top) and Glu179 (bottom) in the water assisted hydrolysis of substrate involving inversion of the anomeric carbon atom.

The enzymatic and physicochemical properties of alpha-amylase and amyloglucosidase enzymes are shown in Table 1.1.

Table 1.1: Properties of alpha-amylase and amyloglucosidase of *Aspergillus niger*.

EC Number	3.2.1.3	3.2.1.1
Name	amyloglucosidase	α -amylase
Systematic Name	glucan 1,4- α -glucosidase	α -1,4 glycan-4-glucanohydrolase
Organism	<i>Aspergillus niger</i>	<i>Aspergillus niger</i>
PDB Code	1AC0	2AAA
Reaction	Hydrolysis of terminal 1,4-linked α -D-glucose residues successively from non-reducing ends of the chains with release of β -D-glucose	Endohydrolysis of 1,4- α -glucosidic linkages in oligosaccharides and polysaccharides
Enzyme Family	Hydrolase	Hydrolase
Enzyme Subfamily	Glycosyl hydrolase (GH)	Glycosyl hydrolase (GH)
Temperature Range	50-65 °C	60 °C
pH Range	4.5	5.5

1.3.2 Glucose Oxidase (GOx)

Glucose oxidase (GOx) also known as beta-D-glucose:oxygen 1-oxido-reductase is a flavoenzyme that belongs to the oxidoreductase enzyme family and is involved in the pentose phosphate pathway. It catalyzes the redox reaction (Figure 1.13) **Glucose + O₂ → D-gluconolactone + H₂O₂**.

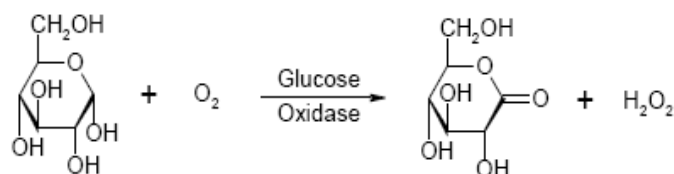


Figure 1.13 : Glucose oxidase reaction. GOx catalyses the oxidation of β-d-glucose by molecular oxygen to d-gluconolactone and H₂O₂. Formation of d-gluconolactone is then followed by the spontaneous hydrolysis of it into gluconic acid.

Various organisms have been identified as GOx producers but only the enzymes from *Aspergillus niger* and *Penicillium amagasakiense* attract commercial attention and studied extensively (Witt et al., 2000) [62]. Glucose oxidase of *A. niger* is a homodimer and requires one flavinadenin dinucleotide (FAD) as a cofactor per monomer. Molecular weight of this homodimer is ~160 kDa. FAD molecule is not covalently bound to the monomer but it is tightly associated. Figure 1.14 indicates *Aspergillus niger* GOx homodimer.

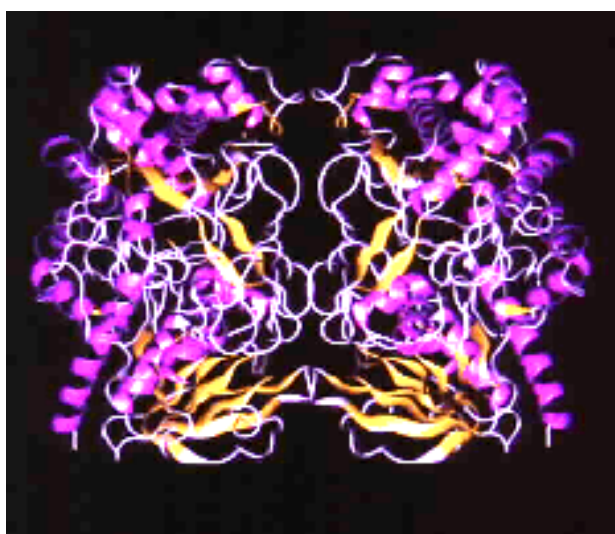


Figure 1.14: 3D-structure of the GOx holoenzyme / homodimer [26].

It is known that GOx reaction proceeds through a Ping-Pong mechanism (Gibson et al., 1964) [19]. The redox reaction could be divided into two half reactions. These half reactions are called reductive and oxidative half reactions. In the reductive half reaction glucose is oxidized to d-gluconolactone and two electrons and one proton are transferred to the flavin ring of FAD and one proton is transferred to the enzyme and at the end d-gluconolactone is hydrolyzed into gluconic acid by one water molecule. As a result, FAD is reduced to FADH₂. In the oxidative part, the transferred electrons and protons are transferred from enzyme to the molecular oxygen and H₂O₂ is produced simultaneously reproducing the oxidized state of the enzyme (Figure 1.15).

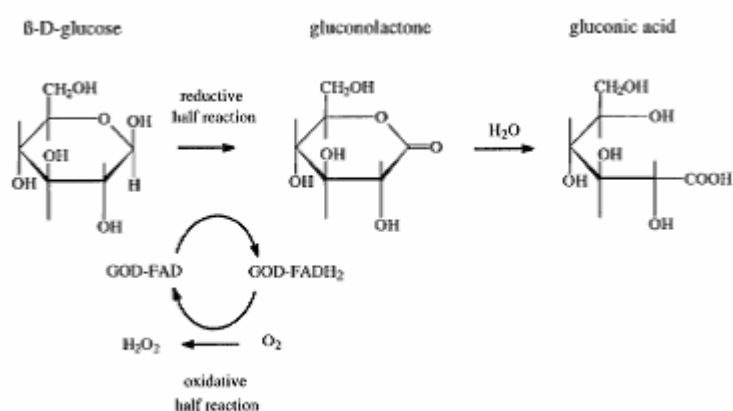


Figure 1.15: Reductive and oxidative half reactions glucose oxidation by GOx [62].

The general scheme for the reductive half reaction obeys general base catalysis. Actually there are two proposed mechanisms. In the first one, a hydride is transferred from glucose C1 to flavin N5 and in the second one, glucose -OH group attacks the flavin C4 nucleophilically and a proton is abstracted from glucose C1 simultaneously. Potential proton acceptor for *A. niger* glucose oxidase is predicted to be His 516/520 (Bright et al., 1964, Weibel et al., 1971, Hecht et al., 1993) [8, 62, 23].

In the oxidative half reaction, binding of molecular oxygen to the active site of the enzyme causes the transfer of the first electron from FADH₂ to the dioxygen molecule resulting in the formation of a superoxide. At low pH, since the active site His residue is protonated, the rate of electron transfer from the dioxygen molecule is increased

through electrostatic or H-bond stabilization and this forms the rate limiting step of the reaction. Then, additional electron and proton transfer steps occur for the production of peroxide.

The glucose oxidase enzyme from *Aspergillus niger* exhibits its optimum catalytic properties at pH 5.5-6.0 and between 40-60 °C. It is also found to preserve 90 % of its catalytic activity at pH 4 and 7 (Cho et al., 1978) [11].

1.3.3 Biopolishing Enzymes-Cellulases

The very first strain of fungi that is capable of hydrolyzing cellulose was first discovered during World War II when it was noticed that the rate of deterioration of cellulosic materials that belong to the U.S. Army was increased in the South Pacific. In order to produce a solution to this problem immediate research was started and as a result the first strain QM6a was isolated. This strain was first identified as *Trichoderma viride* and later recognized as *Trichoderma reesei* (Bhat et al., 2000) [4]. The research on this cellulose degrading organism and cellulose degradation has started. In the last half of the 20th century there has been a remarkable progress in isolation of microorganisms producing cellulases; improving the yield of cellulases by mutation; purifying and characterizing the cellulase components; understanding the mechanism of cellulose degradation cloning and expression of cellulase genes; determining the 3-D structures of cellulase components; understanding structure-function relationships in cellulases; and demonstrating the industrial potential of cellulases [37].

Cellulolytic enzymes are produced by a wide variety of organisms. Few of these enzymes are capable of degrading crystalline cellulose effectively. Among these microorganisms, the extremophilic ones are very important because of the stability of their enzymes under harsh conditions such as highly acidic and alkaline pHs as well as temperatures up to 90 °C (Lamed et al., 1988) [38]. Important thermophilic microorganisms capable of degrading cellulose are *Clostridium thermocellum*, *Thermomonospora fusca*, *Thermoascus aurantiacus*, *Sporotrichum thermophile*, *Humicola insolens* and *Chaetomium thermophile*. *Clostridium thermocellum* differs

from other cellulolytic microorganisms since it secretes all its cellulolytic enzymes in a protein complex called cellulosomes (Nemeth, A., 2002) [47]. Most extensive research about the cellulases has been done on aerobic fungi such as *Trichoderma koningii* (Halliwell et al., 1981) [22] and *T. reesei* (Miettinen-Oinonen et al., 2005, Liming et al., 2004, Medve et al., 1998) [44,41,43].

Cellulases are the only enzymes used in biofinishing of the cotton fabrics. These enzymes are suitable for wet processes and they can be used almost in all textile machines. Nowadays commercial cellulase preparations for different types of fabrics are available for use in biopolishing. They exhibit a wide range of pH and temperature stability and activity. Commercial cellulase preparations are mostly from the filamentous fungi, *Trichoderma reesei*. Cellulases are extracellular enzymes. They are secreted out of the cells. Industrial producers take this advantage into consideration. That is why *Trichoderma reesei* is the workhorse of industry in terms of production of cellulases. *Trichoderma reesei* produces cellulases in large quantities and secretion of the enzyme components allows rapid purification of the enzymes.

Researchers are also working on producing genetically modified cellulase enzymes with the desired properties for different types of processes. Directed evolution and site directed mutagenesis studies which target the cellulases are reported [53]. Moreover, site-directed mutagenesis studies have been used for the identification of active site residues and residues responsible for the stability of the cellulases. These studies are very valuable tools because they provide the information for the design of new cellulases having specific activities.

Cellulases are multicomponent enzymes. There are three major types of cellulases secreted by *Trichoderma reesei*: Endoglucanases, 1,4- β -D-glucan 4-glucanohydrolases; Cellobiohydrolases, 1,4- β -D-glucan cellobiohydrolases; Cellobiases, β -D-glucosidases [48]. *Trichoderma reesei* has at least six endoglucanases, two cellobiohydrolases, and two β -D-glucosidases (Bhat et al., 1997, Heikinheimo, L., 2005) [3,25]. Figure 1.12 indicates the molecular weights and number of aminoacids of some of the cellulase components.

Table 1.2: Molecular weights and number of aminoacids of *Trichoderma reesei* cellulases (Vinzant et al., 2001) [60].

Cellulase components of <i>Trichoderma reesei</i>	Molecular Weight (kDa)	Number of amino acids
EG I	48,2	459
EG II	44,2	418
EG III	23,5	218
EG IV	35,5	344
EG V	24,5	242
CBH I	54	513
CBH II	49,6	471
B-D-glucosidase I	78,5	744

Cellulases belong to the glycosyl hydrolase family of enzymes. This enzyme family contains nearly 96 subfamilies. Cellulases are present in at least 12 of these subfamilies [63]. Subfamily classifications of the glycosyl hydrolase family are done on the basis of their amino acid sequences. The three dimensional structures and enzyme-substrate interaction mechanisms display some differences in every subfamily.

These multicomponents act synergistically for the degradation of cellulose. They act specifically on 1,4- β -glycosidic bonds of the cellulose. Cellulase has two domains. One of them is the catalytic domain and the other is the cellulose binding domain. These two domains linked by a short linker peptide forms the intact bimodular enzyme (Sandgren, M.C., 2005, Kleywegt, G.J., 1997) [53, 36]. The linker peptide is rich in Proline, Threonine and Serine residues and it is often O-glycosylated. O-glycosylation provides maintenance of the extended conformation of the linker peptide and also protects the linker region against proteases.

Cellulose binding domains of fungi, algae and bacteria are classified into two families. The shorter CBDs (30-40 amino acids) are the ones from the fungi are classified as family II and the longer ones (100-150 amino acids) are the ones from bacteria and algae and classified as family I (Reinikainen et al., 1995) [51]. They are thought to have arisen by a convergent evolution since they do not have much sequence

similarities. But they have conserved aminoacids having aromatic side chains and these are thought to be involved in cellulose binding in all types of CBDs. Much effort has been put to clarify the mechanism of adsorption and its effect on the activity of the cellulase components. Deletion mutants of cellulase components (CBD deleted) were prepared and analysis of their adsorption trends revealed a decrease of 50-80 % of activity of fungal cellulases on insoluble substrates (Reinikainen et al., 1995) [51].

It was suggested that in CBHI of *T. reesei* both core domain and CBD participated in the binding and in bacteria only CBDs are involved in binding. Site directed mutagenesis directed towards CBDs of CBHI (Y492A, Y492H and P477R) indicated that conserved aromatic aminoacids are essential in binding [51] and it is known that hydrophobic interactions are also important for binding (cited in Reinikainen et al., 1995).

1.3.3.1 Endoglucanases, (EG)

(endo-1,4- β -gluconase, 1,4- β -D-glucan-4-glucanohydrolase, EC 3.2.1.4)

Endogluconases are the endocellulases which randomly hydrolyze the cellulose chains internally. Action of endoglucanases produces new chain ends and changes the degree of polymerization of the cellulose. There are at least 6 identified endoglucanases in *Trichoderma reesei* (EG I-VI). EG VI was described only at the protein level (Bower et al., 1998) [6]. EG I and EG II are the main components of the *T. reesei* endoglucanases and they comprise ~10 % of the secreted proteins of the organism (Heikinheimo et al., 2001) [24].

EG I, EG II and EG III cleave β -1,4-glycosidic bonds with retention of anomeric configuration, yielding the β -anomer as the reaction product and EG IV uses the inverting mechanism. The exact mechanism of EG V and VI is not exactly known according to the current knowledge. Comparison of the structures of the *T. reesei* EG I and *H. insolens* EG I, reveals that they have similar substrate-binding grooves: both proteins have their active site located in an open cleft (Sandgren, M.C., 2005) [53]. Figure 1.16 shows the three dimensional structure of Endoglucanase I of *T. reesei*.

The target of the endoglucanases is the amorphous cellulose (Heikinheimo et al., 2001) [24]. They exhibit lower activity towards insoluble substrates such as crystalline cellulose.



Figure 1.16: 1EG1, The crystal structure of the catalytic core domain of endoglucanase I from *Trichoderma reesei* at 3.6 Å resolution (Klegwegyt, G.J., 1997) [36].

Azavedo et al.(2000), suggested that agitation levels has a profound effect on endoglucanase activity and at high levels of agitation the presence of CBDs is not key to the functioning of the endoglucanases [2].

1.3.3.2 Cellobiohydrolases (CBH)

(exo-1,4- β -glucanase, 1,4- β -D-glucan cellobiohydrolase, EC 3.2.1.91)

Cellobiohydrolases are exocellulases that hydrolyze the cellulose chains from the ends releasing cellobiose as the end product. *T. reesei* has two CBHs. CBH I splits cellobiose from the reducing end and CBH II from the nonreducing end (cited in Heikinheimo, L., 2002) [25].

Structural studies revealed that CBH I core domain contains a 40 Å long tunnel shaped active site along the enzyme molecule. This tunnel shaped active site explains the high affinity of CBHs to crystalline cellulose during the progressive catalytic cycles. This also explains the processivity seen in CBHs. The loops present on the surface of CBH allows the extricated cellulose chain from adhering back to the crystalline

cellulose. Moreover, since the crystalline cellulose is the highly ordered one, it can fit easily into that tunnel whereas amorphous cellulose having a more loose structure can not easily fit to the same cavity. Figure 1.16 indicates the three dimensional structure of CBH I from *T. reesei*. CBH I has a retaining mechanism of hydrolysis whereas CBH II has inverting mechanism (Sandgren, M.C., 2005) [53].



Figure 1.17: 1CEL (EC 3.2.1.91). The three-dimensional crystal structure of the catalytic core of cellobiohydrolase I from *Trichoderma reesei* (Divne et al., 1994) [15].

1.3.3.3 β -glucosidase

β -glucosidases also called cellobiases are responsible for the hydrolysis of cellobiose produced by CBH enzymes into glucose. The function of β -glucosidase is very important. It is known that CBH and EG are inhibited by cellobiose (Gruno et al., 2004) [50]. So the main function of the β -glucosidase in the cellulase system is to overcome the product inhibition of CBH and EGs (Lenting et al., 2001) [40]. The three dimensional structure of *T. reesei* β -glucosidase is not solved yet.

There exists a strong synergism between cellulase components. The types of synergisms reported upto now are endo-endo, endo-exo, exo-exo, endo-exo-glucosidase exo/endo-glucosidase. However, endo-exo synergism is the most extensively studied one. There is also an intramolecular synergy between the CBD and core catalytic domains. The degree of synergism is different for each type of substrate. For example, endo-exo synergism is mostly pronounced for the degradation of the crystalline cellulose. Degree of synergism was found to be most for cotton and then Avicel and

least for acid swollen amorphous cellulose (Zhang et al., 2004) [64]. Figure 1.18 shows the synergistic action of cellulases on cellulose. In this synergism, endoglucanases adsorb to the cellulose microfibrils and start to make internal cuts in the cellulose chain. Then cellobiohydrolases start the hydrolysis from the newly created chain ends and CBHs hydrolyze the cellulose chain processively. This combined action increases the efficiency and activity of the whole cellulase system with respect to the enzymes acting alone.

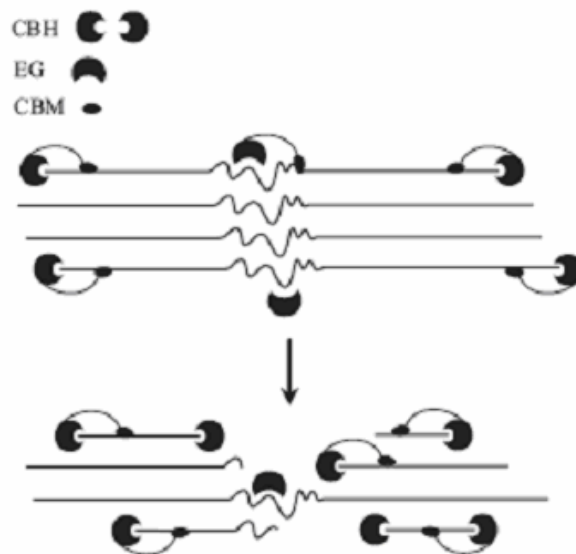


Figure 1.18: Endo-exo synergism between endoglucanases and cellobiohydrolases/exoglucanases during cellulose hydrolysis [25].

CHAPTER 2

IMMOBILIZATION OF ENZYMES

2.1 Why to immobilize?

Immobilization of biomolecules on solid supports has been gaining importance over the last 40 years. The developments in the biosensor technology and in the enzyme technology enable the researchers to find different supports suitable for different applications. Immobilization of biomolecules such as enzymes offers many advantages. Most important ones are increased stability and durability, multiple use, longer half-life. Generally, binding an enzyme onto a solid support increases the stability of the enzyme by the multipoint attachment of the enzymes onto the support. Multipoint attachment of the enzyme and also the immobilization procedure itself may reduce the activity of the enzymes because overloading of the enzymes onto the support matrix and on top of each other may restrict the enzyme-substrate interaction mostly by limiting the access of substrates to the active site due to steric hindrance/clashes or by actual closure of the active site of the enzyme (Edt. Bickerstaff, 1997) [16].

For the immobilization of enzymes the first consideration is the choice of the support material suitable for the application and the method for immobilization. The properties of the support material as well as the enzyme's intrinsic properties determines this choice and the method to be used. Factors taken into consideration for the choice of the supports are

- Physical characteristics (granules, sheets, inner tube walls)
- Mechanical properties (rigidity and durability, resistance to mechanical agitation and compression)
- Resistance to chemicals and microbial activity
- Tendency to incorporate water into its structure, hydrophilicity (important for the preservation of the enzyme in a highly active state)
- Permeability / porosity
- Price and availability (Colowick et al.,1976) [14].

2.2 Methods

There are five well-known methods of immobilization. These are adsorption, entrapment, covalent coupling, membrane incorporation and crosslinking. Actually crosslinking is not a type of immobilization, but its a method where there is no support that the enzyme of interest is immobilized. Rather enzyme molecules are crosslinked to each other via specific aminoacid reactive groups with the use of a crosslinker. But most of the authors classify crosslinking under immobilization (Edt. Bickerstaff, 1997) [16].

2.2.1 Adsorption

It is the simplest method of all. Reversible surface interactions are formed between the enzyme and the support material. These interactions are relatively weak but intense in number. Enzyme molecules adsorb onto the support via electrostatic forces. Of these electrostatic interactions, Van der Waals, ionic and H-bonding interactions are important. In this method, the surface chemistry of the enzymes and the support matrix is exploited. This method forms the basis of many chromatographic methods such as ion-exchange, affinity, adsorption and hydrophobic chromatographies.

First a support with suitable adsorption capabilities is chosen. Ion-exchange matrices like diethylaminoethyl (DEAE) or carboxymethyl cellulose (CMC) or DEAE-

and CM-dextran (DE-Sepharose and CM-Sepharose[®]) are very suitable supports for adsorption. Hydroxylapatite or calcium phosphate adsorption materials, polymeric resins, porous carbon could also be used. Most important factor for the adsorption of a protein onto a support is the ionic strength and pH (Bisswanger, H., 2004) [5]. At low ionic strength binding is favored and at high ionic strength binding is weakened. It is a reversible method and the adsorbed protein could be eluted using high ionic strength buffers. Since there is no ideal recipe for the immobilization procedures this is same for the adsorption procedures. The reversibility of binding of the enzymes allows the researchers to find the suitable conditions for the adsorption. The advantages of adsorption are:

- damage to the enzymes/ protein is little
- simple and quick
- chemical modification of both enzyme and the support material is avoided
- reversible.

There are also disadvantages offered by this method. These are:

- leakage of the enzymes from the support
- nonspecific binding
- enzyme overloading on the support
- steric hindrance.

Leakage is the most important disadvantage since it directly affects the yield. Desorption of the enzyme from the support matrix could be accelerated by the changes in ionic strength, pH, temperature. Moreover, enzymes could easily be desorbed from the support as a result of the binding of the substrate, the products and the contaminants present in the reaction mixture or as a result of the physical factors such as agitation, particle-particle abrasion, flow rate during the reaction. Sometimes, the reaction causes a change in the three dimensional structure of the enzyme and this event may also promote desorption. Nonspecific binding may also create a problem if the substrate, the contaminants or the product have an affinity for the support material. This may eventually result in the alteration of reaction kinetic parameters such as V_{\max} and K_M .

Activity losses may result from the overloading of the support or the steric hindrance by the support.

2.2.2 Entrapment

Enzymes can be trapped inside the polymeric network of the gels such as agarose, gelatin, polyacrylamide and alginate. The enzyme molecules movements are restricted inside the gel due to the lattice structure of the gel. This method differs from other methods in that the enzyme molecules are not bound to the surface but rather they are free in solution and confined to a matrix. Since the enzyme is restricted inside the gel, the diffusion rate of the substrate in that particular network of gel gains importance. Enzymes with low molecular weight substrates are suitable for this method. The porosity of the gel material should be carefully controlled to provide a free space for the enzyme molecules for the catalysis and for the substrates and products for free movement. The gel lattice should also be tight enough/ the pore sizes should be appropriate enough to prevent the leakage of the enzyme molecules. There are several methods used for entrapment that is described by Bickerstaff in Immobilized Enzymes and Cells:

- “- Ionotropic gelation of macromolecules with multivalent cations (such as alginate).*
- Temperature induced gelation (such as agarose, gelatin.)*
- Polymerization by chemical/photochemical reaction (such as polyacrylamide).*
- Precipitation from an immiscible solvent (such as polystyrene).” [16]*

The pore size of the gels as well as its mechanical properties can be determined by changing the amounts of the monomer and the crosslinker. The crosslinker molecule introduces crosslinks between the polymer chains and this helps the formation of the three-dimensional network of the gel.

2.2.3 Covalent coupling/binding

As the name implies, in this method covalent bonding occurs between the enzyme and the support material. More specifically bonding happens between the functional groups present on the surface of the support and the functional groups (mostly side chains) of the aminoacids of the enzyme. Table 2.1 indicates the functional groups of the aminoacids. Since covalent bonds are strong, they can resist harsh conditions so this minimizes the leakage of the enzymes from the support.

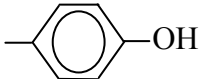
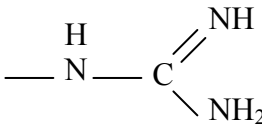
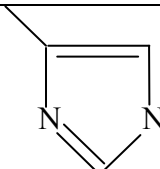
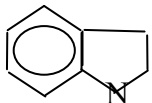
2.2.3.1 Supports

Choice of a suitable support material for the enzyme to be immobilized is an important factor. Generally, the supports are chosen to be hydrophilic. Hydrophilicity of the support enables maintenance of the enzyme activity in the support environment. Moreover, supports with hydroxyl groups form hydrogen bonds with water molecules and this also helps to keep the enzyme molecules in a hydrophilic environment. The criteria for the supports used for covalent binding according to Henry Bisswanger in Practical Enzymology [5]:

- The support must be compatible with the enzyme
- It should contain reactive groups for the attachment of the protein
- It should possess a high binding capacity
- It must be inert to the reaction components
- It must endure long term continuous reaction processes.

Common supports include silica and alumina, glass, ceramics and synthetic polymers such as polyamide, polystyrene, polyacrylate, polyacrylamide etc. they are important because they can resist harsh conditions such as high pressure.

Table 2.1: Reactive residues of proteins [14].

Reactive group	Amino acid	Representation
ϵ -amino	Lysine	-NH ₂
N-terminal amino	All aminoacids	
Sulfhydryl	Cysteine	-SH
Carboxyl	Aspartate, glutamate	-COOH
C-terminal carboxyl	All amino acids	
Phenol	Tyrosine	
Guanidino	Arginine	
Imidazole	Histidine	
Disulfide	Cystine	-S-S-
Indole	Tryptophan	
Thioether	Methionine	CH ₃ -S-
Hydroxyl	Serine, threonine	-CH ₂ OH

2.2.3.2 Surface Modifiers

Surface activation renders the functional groups present on the surface of the support more electrophilic. So the support's functional groups become ready to react with the strong nucleophilic groups of the enzyme. The covalent bond forms as a result of the reaction of these electrophiles and nucleophiles. Specific chemicals are used as surface activators. The type of these chemicals is determined according to the support matrix properties. For example, free hydroxyl groups present on polysaccharide

supports are activated by CNBr and a isourea linkage is formed upon the addition of the enzyme. Figure 2.1 indicates CNBr activation of sepharose [33]. If the support material contains -COOH group on the surface then it can be activated using carbodiimide activation. Figure 2.2 shows this type of activation [33]. Peptide bond is formed between the enzyme and the activated support. If the support contains aromatic amino functional groups, nitrous acid could be the choice of activator and diazo linkage is formed between the enzyme (especially tyrosine like aminoacids containing ring structure forms the diazo linkage) and the support.

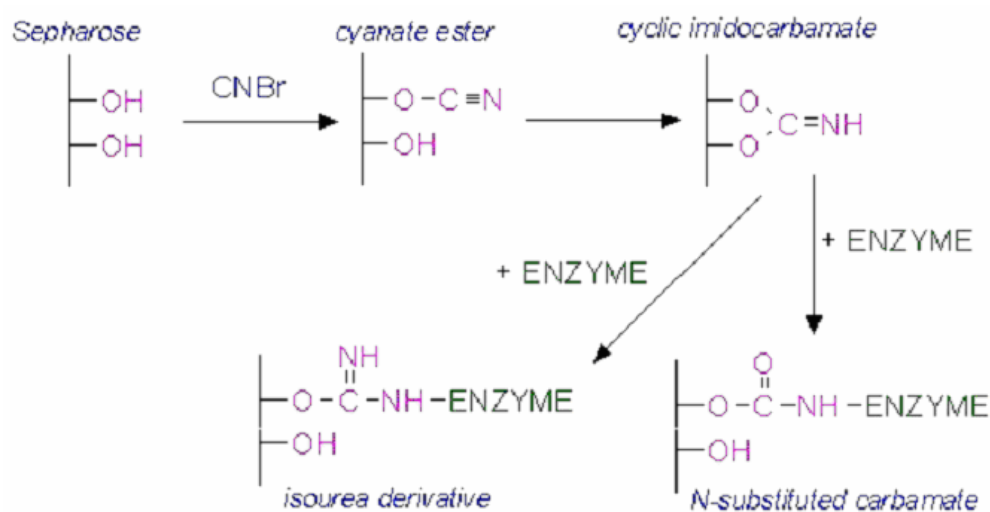


Figure 2.1: Sepharose activation by CNBr. Formation of an isourea and N-substituted carbamate derivatives [33].

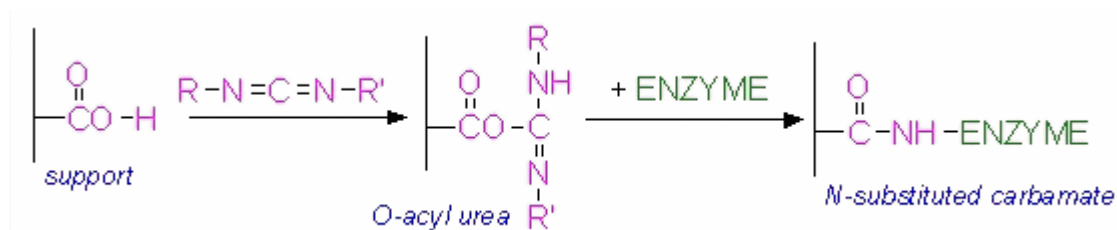


Figure 2.2: Carbodiimide activation of a support with carboxyl group [33].

The choice of the method (type of linkage) for covalent bond formation is also an important factor affecting yield of the whole procedure. Care should be taken to avoid the covalent bond formation between the active site residues of the enzyme and the support material. Participation of active site residues in covalent bond formation results in blocking of the active site and activity losses as expected. Therefore survival of the

active site upon coupling gains importance in the choice of the support and the coupling method to be used.

Glass, as a support can resist to strong acids and solvents and it has a compact and solid structure. These physical properties makes glass a very valuable material for the immobilization of enzymes in many technical processes. However there are some drawbacks of these type of supports. First, glass has an inert nature and because of its compactness, accessible surface area is relatively low. So silanization with different types of silanes is used to introduce reactive groups to glass supports. Most commonly used silanes are:

- 3-aminopropyltriethoxysilane (APTS): This silane introduces amino groups onto the glass surface by reacting with the hydroxyl groups on glass (Figure 2.3).

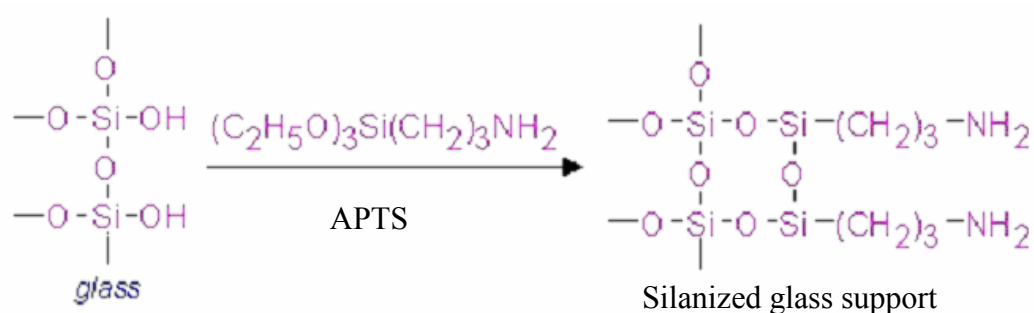


Figure 2.3: Silanization of glass with APTS [33].

- 3-Glycidoxypentyltrimethoxysilane (GPTS): GPTS (Figure 2.4 shows the structure of GPTS) introduces epoxy groups on the surface of the glass by also reacting with free hydroxyl groups. The opening of the epoxy ring in acidic medium provides a reactive group to which the enzyme reactive groups can bind.

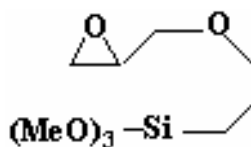


Figure 2.4: Structure of 3-Glycidoxypentyltrimethoxysilane (GPTS).

2.2.4 Encapsulation

In this method, enzyme solution is restricted in small vesicles having porous membranes. Encapsulation is very similar to entrapment in that the enzyme molecules are free to move in solution but can not diffuse in and out of the capsule. Generally semipermeable membranes and hollow fibers are used for encapsulation of the enzymes. These membranes and hollow fibers should allow the passage of substrates and the products and they should not be permeable to the enzyme molecules [43,48].

The simplest version of encapsulation is the use of dialysis bags. This application is not very efficient due to the large diffusion distances and small surface area of the membrane and diffusion related problems. To overcome this problem microcapsules made from nylon and nitrocellulose are frequently used for the microencapsulation of the enzyme molecules. Enzymes are also encapsulated in liposomes in solution. This method is usually used for drug delivery since most of the drugs have to pass the plasma membrane to reach their targets. Liposomes and the plasma membrane are very similar in structure and liposomes can fuse with the plasma membrane and release the drug into the cytoplasm of the target cells.

Entrapment is a recommended technique when the enzyme of interest is very sensitive and has to be kept intact. Another application includes the coimmobilization of different enzymes to perform sequential reactions. This may be quite useful for industrial processes.

2.2.5 Cross-linking, CLECs and CLEAs

No support material is used for crosslinking. Instead, enzymes are crosslinked to each other. Crosslinking of the enzymes causes their aggregation and helps their recovery from the solution. Mostly, two types of crosslinkers are used in this method. Homobifunctional crosslinkers are the ones that bind to the same reactive groups on both sides and heterobifunctional crosslinkers are the ones that have the capability of binding to different reactive groups on each side. Multifunctional crosslinkers are also available for use. For example glutaraldehyde is a homobifunctional crosslinker and forms oligoglutaraldehyde in solution (Figure 2.5).

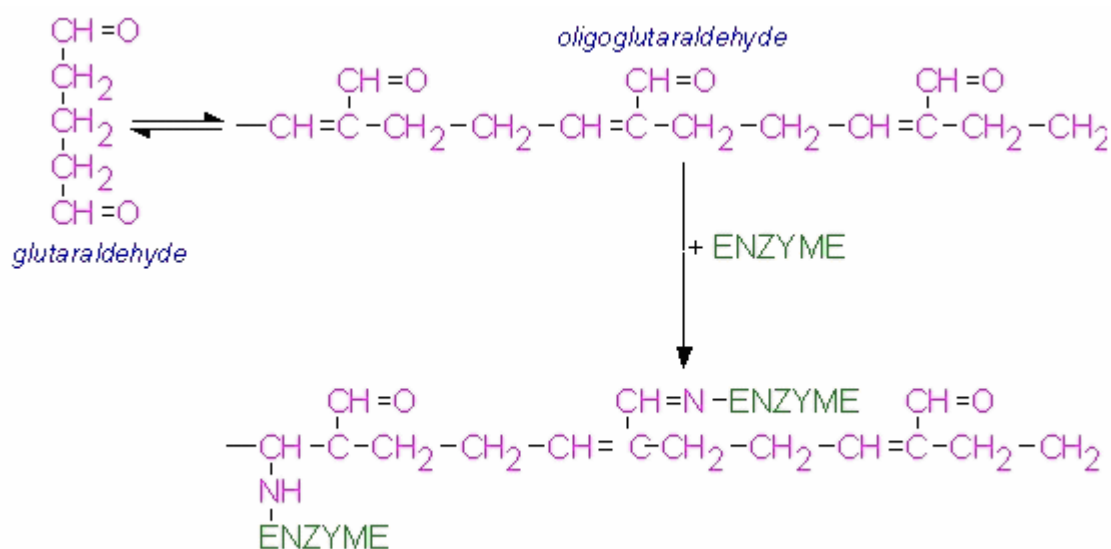


Figure 2.5: Crosslinking of an enzyme with a homobifunctional crosslinker, glutaraldehyde [33].

It is known that glutaraldehyde reacts with ϵ -amino groups on Lysine residues and also N-terminal amino groups (Richards and Knowles, 1968 cited in Methods in Enzymology, v. 44) [14]. This reaction is through the double bonds of its oligomeric

form. It can not be a single bond since it is very stable and formation of a simple Schiff-base can not provide that stability. Moreover, freshly distilled solutions of glutaraldehyde indicates lower reactivity on proteins. Glutaraldehyde is widely used in crosslinking because of technical ease and versatility of its application.

Crosslinked enzymes form a large, three-dimensional complex structure. Since the crosslinking attaches all the enzymes together, reduced activity or stability due to the steric hindrance is expected. Introduction of spacer molecules or proteins such as Bovine Serum Albumin (BSA) may be a solution to the close proximity problems.

Nowadays, the activity losses as a result of crosslinking is overcome by the introduction of Crosslinked enzyme crystals (CLEC) and Crosslinked enzyme aggregates (CLEA) technologies. Since it is hard to get enzymes as crystals CLEC is not used very much but CLEA technology (Figure 2.6) is much more simple and proven to cause hyperactivation of glucose oxidase, laccase, lipase enzymes (Sheldon et al., 2002) [55].

CLEAs are prepared by precipitating the enzyme molecules by a polar precipitant solution such as ammonium sulphate, ethyl lactate, PEG, tert-butyl alcohol etc. and then crosslinking these aggregates with a suitable crosslinker (mostly glutaraldehyde). It is shown that formation rate of aggregates increases as the polarity of the solvent increases. Use of CLEAs is a universal and cheap alternative to other crosslinking methods because pure proteins with enhanced activity and having higher protein ratios per volume is reached.

CHAPTER 3

PURPOSE

This study includes the results of two projects of TUBITAK Textile Research Center (TAM 2003-06 and TAM 2003-08). Enzyme are replacing the use of harsh chemicals during the textile processes because they are specific and they are offered as environmentally friendly alternatives.

Viscose knitted fabrics are more prone to pilling than any of the other fabric types. Generally the cellulase enzymes used in the biopolishing of Lyocell and cotton are used to remove those pills on the surface of the viscose fabric but according to our current knowledge these commercial cellulase formulations are unable to remove the pills effectively. There are no cellulase formulations that effectively removes the pills on viscose knitted fabrics. Moreover, action of the cellulases cause loss of fabric strength due to the damage in the highly ordered crystalline regions of the viscose fibers. In the first part of the study, commercial cellulases used in the biopolishing of the viscose fabrics were crosslinked in an attempt to minimize the loss of fabric strength and to enhance the removal of the pillings.

Use of commercial H_2O_2 is being replaced by the use of enzymatically produced H_2O_2 for textile bleaching. The glucose produced during desizing process of the cotton fabrics by amyloglucosidases is used for the enzymatic production of peroxide and then this peroxide is used for the bleaching of the cotton fabric. One advantage of this combined system is the use of one process bath instead of two separate process baths. Most important problem in this combined system is the insufficient peroxide and

whiteness levels reached due to the impurities present in the desizing and bleaching baths and it is hard to find suitable enzymes that can work without affecting catalysis of each other. Another problem arises from the fact that use of enzymes would bring an additional cost to the system. In order to reduce the costs, enzymes can be recycled by immobilization. In the second part of the study, commercial GOx enzyme is immobilized on different supports with an effort to allow enzyme recycling and to increase the efficiency of the enzymes. GOx and immobilized GOx were used to produce H_2O_2 from the desizing liquor for the bleaching of cotton.

CHAPTER 4

MATERIALS AND METHODS

4.1 Materials

4.1.1 Chemicals

Name of the Chemical	Supplier Company	Catalog Number
30% Acrylamide-0.8% Bisacrylamide solution	Biorad	161-0158
Acetic acid	Riedel de Haen	27225
Al₂O₃	Acros Organics	-
Ammonium persulphate	Riedel de Haen	11222
Ammonium sulphate	Riedel de Haen	11225
APTS	UCT Chemicals	A0750
BSA	Sigma	A7906
BTCA	Bayer	
Carboxymethylcellulose (substitution 0.75-0.85, viscosity ~100)	Aciselsan	ASEL SY100
CNBr activated Sepharose-4B	Sigma	C9142
Congo Red	Delta	-
Coomassie Brilliant Blue CBB-G250	Merck	115444
D-(+) Glucose	Sigma	G-7021
Dinitrosalicylic acid	Fluka	42260

Dipotassium hydrogenphosphate	Riedel de Haen	04248
EDTA	Sigma	E 5134
Ethanol	Riedel de Haen	32221
Glutaraldehyde (25%)	Merck	1.04239
Glycerol	Riedel de Haen	15523
Glycine	Sigma	G-8790
GPTS	UCT Chemicals	G6720
H₂SO₄	Riedel de Haen	30743
HCl	Merck	100314
Jeffamine ED-2001[®]	Merck	65605-36-9
KMnO₄	Merck	5080
Liquid nitrogen	Karbogaz	-
Methanol	Riedel de Haen	24229
Na-K tartarate tetrahydrate	Riedel de Haen	25508
NaOH	Merck	106462
PEG 6000	Fluka	81253
Potassium dihydrogenphosphate	Riedel de Haen	4243
Prestogen SP	BASF	-
Rucogen WBL	Rudolph Duraner	-
SDS	Sigma	L4390
Sephadex G50	Amersham	
Silica (60-200 mesh, 100 Å)	ICN	O3220
Sodium acetate three hydrate	Riedel de Haen	32318
Sodium bicarbonate	Laborlar Kimya	
Sodium chloride	Riedel de Haen	
TEMED	Sigma	T7024
Triethanolamin (triethylamin)	J.T. Baker	8079
Tris	Fluka	93349
Tris-HCl	Applichem	A3452
Triton X-100	Applichem	A1388
Tween 20	Merck	822184
Tween 80	Merck	

4.1.2 Enzymes

Enzyme Name	Supplier Company	Origin
Inotex AG (amyloglucosidase)	Inotex	<i>A. niger</i>
Gempil 4L	Gemsan	<i>T. reesei</i>
Glucoseoxidase	Biozymes	<i>A. niger</i>
	Novozymes	<i>A. niger</i>
IndiAge 2XL	Genencor	<i>T. reesei</i>

4.1.3 Solutions

4.1.3.1 SDS-PAGE Solutions

- 3M Tris-HCl pH 8.9
- 1M Tris-HCl pH 6.8
- 10 % (w/v) SDS
- 10 % (w/v) APS
- TEMED stock solution
- 30% Acrylamide-0.8% Bisacrylamide stock solution
- 10X denaturing running buffer (SDS-Tris-Glycine, 1:2.9:14.4 (weight ratios))
- 1X denaturing running buffer (pH 8.3)
- 1 % (w/v) Coomassie Brilliant Blue solution in (filtered)
- Staining Solution (1% Coomassie Brilliant Blue, 50 % Methanol, 10 % Acetic acid, ddH₂O)
- Destaining solution (5 % Methanol, 7 % Acetic acid, ddH₂O)
- 2 x SDS Sample Buffer (loading buffer) (reducing) (used 1:1) (80mMTris/HCl pH 6.8, 10 % Mercaptoethanol, 2 % SDS, 10 % Glycerin, ddH₂O)

4.1.3.2 Buffers

- a) 0.05 M NaOAc pH 4.8.
- b) 0.1 M NaOAc pH 8.42.
- c) 0.1 M NaHCO₃/ 0.5 M NaCl (pH 8.3-8.5).
- d) 1 M Tris pH 8.
- e) Triethanolamin pH 8.
- f) 1.25 M pH 8 Potassium phosphate buffer.

4.1.3.3 Other Solutions

- a) 1 % CMC in 0.05 M NaOAc buffer, pH 4.8.
- b) DNS reagent (1 % DNS, 1.6 % NaOH, 30 % Rochelle's Salt in ddH₂O (all w/v)).
- c) 1 % DNS solution .
- d) 40 % Rochelle's salt.
- e) 0.2 M Glycine pH 8.
- f) 5X Bradford Reagent stock (0.5 g Coomassie Brilliant Blue G250, 25 % Methanol, 42.5 % H₃PO₄ in ddH₂O) (Working solution: 1X).
- g) 10 % APTS solution in 50 % Ethanol.
- h) Glucose standards in 0.05 M NaOAc pH 4.8 (0.1 M, 0.01 M, 0.007 M, 0.004 M, 0.002 M, 0.001 M).
- i) BSA standards in ddH₂O and in 0.05 M NaOAc (0, 0.05, 0.1, 0.2, 0.4, 0.5, 1 mg/ml).
- j) 0.25 M, 0.5 M, 1 M Sodium phosphate pH 7.
- k) 1.25 M Potassium phosphate pH 8.
- l) 4 M, 3.3 M, 2 M, 1M (NH₄)₂SO₄ in 0.1M pH 8 Potassium phosphate buffer
- m) 2.5 % glutaraldehyde with 1 % (v/v) phosphoric acid (the pH is adjusted to 7.3 with NaOH).
- n) 0.1 M Sodium borohydride.

4.1.4 Fabric Properties

4.1.4.1 Cotton

Fabric: 175 g/m², plain cotton fabric, 13 weft/cm- 14 warp/cm

Starch size ratio: 100 %

Before enzymatic bleaching desizing process with amyloglucosidase was applied to the fabric and the bleaching process was combined with desizing.

4.1.4.2 Viscose

Fabric density: 137 g/m²,

100 % viscose supreme (single Jersey) knitted fabric

Bleached and primary fibrillation was performed

4.2 Methods

4.2.1 Biopolishing and Crosslinking

All activity tests and fabric trials were performed in 0.05 M pH 4.8 NaOAc buffer at 55 °C unless otherwise stated.

4.2.1.1 Endogluconase Activity Test

The activity test was performed using Miller's reducing sugar test [45] also called Dinitrosalicylic acid (DNS) Method with minor differences. and 1% carboxymethyl cellulose (CMC) was used as the substrate. Two blanks and triplicates of each sample were prepared. Lyophilized samples were dissolved in 0,05 M NaOAc buffer (pH 4.8).

Preparation of Blanks: 60 µl of enzyme for each sample was incubated at 100°C for 1-2 min. Then 300 µl DNS reagent was added. The solution was mixed well and 600 µl 1% CMC was added. The tubes were incubated at 100°C for 10 min. Then they were put on ice immediately.

Preparation of Samples: : 60 µl of each enzyme was preincubated at 50°C for 5 min. 600 µl 1% CMC was added and vortexed. The tubes were incubated at 50°C for 10 min. The reaction was terminated using 300 µl DNS reagent. After the addition of DNS reagent samples were reincubated for 10 min. at 100°C. Then they were placed on ice immediately.

Preparation of glucose standards: 5 standards (1:10,1:15,1:25,1:50,1:100) were prepared by serial dilutions from 0,1 M glucose in NaOAc buffer. 60 µl from each standard were put into the corresponding tubes. 300 µl DNS reagent and 600 µl 1% CMC were added. Tubes were boiled at 100 °C for 10 min and placed on ice immediately. 60 µl NaOAc was used instead of the enzymes in the blanks. 300 µl DNS reagent and 600 µl 1% CMC were added. Tubes were boiled at 100 °C for 10 min and placed on ice immediately.

All the samples were diluted properly in order to put the measurements in the linear range of the spectrophotometer. The absorbances were measured at 540 nm for Blank 1 and Blank 2 of each sample and the mean values were taken in the calculations. The standard curve was constructed from glucose standards with $R^2 > 0.90$. Throughout all the experiments, the same glucose standard curve is used for pH 4.8 experiments and for pH stability experiments standards curves for different pHs were prepared by

dissolving 1 % CMC in buffers of different pHs. 1 CMC unit is defined as the enzyme that produces 1 mol glucose from carboxy methyl cellulose per minute.

Activity Calculations:

$$\text{CMC IU/ml} = (1/m \times \Delta A_{540} + C_0) \times \text{DF} / (t \times \text{MW}_{\text{glucose}})$$

m=slope of the standart curve

$$C_0 = m_{\text{glucose}} \text{ for the unkown sample(mg)} \quad C_0 = M_{\text{glucose}} \times V_{\text{rxn}} \times \text{MW}_{\text{glucose}}$$

DF=Dilution Factor

t=reaction time(min)

$$\text{MW}_{\text{glucose}} = 0,18016 \text{ mg}/\mu\text{mol}$$

1/m=13,587 (reciprocal of the slope of the glucose standard curve prepared at pH 4.8 0.05M NaOAc)

$$V_{\text{rxn}} = 0,96 \text{ ml or } 0.48 \text{ ml}$$

4.2.1.2 [Protein] Determinations

Protein concentrations were determined by Bradford protein assay [7] and using BSA as the standard. BSA standards have the concentrations of 0, 0.05, 0.1, 0.2, 0.4, 0.5, 1 mg/ml BSA. Protein concentrations more than 1 mg/ml are out of the range of the assay. Proper dilutions of the samples and standards were prepared accordingly. 10 µl of each sample and standard are put into 96-well microtiter plates and 200 µl. of diluted (1/5) Bradford reagent was added. After 5 minutes of incubation in dark and at room temperature, the readings were taken at 595 nm using Biorad Microtiter Plate Reader. The protein concentrations of the samples are determined using the BSA standard curve. Each time standard curves having $R^2 > 0.90$ are used in the calculations.

4.2.1.3 Crosslinking Methods

Cellulase Crosslinking with Jeffamine:

1X volume of Jeffamine (from 10 % w/v) was added to 2X volume of commercial cellulases (Baylase, Gempil 4L Concentrated, IndiaAge 2XL). The total reaction volume was 100X. The reactions were done in ddH₂O. Small scale reactions were performed in 50 ml. Falcon tubes and large scale reactions in Erlenmayer Flasks. Proper amount of glutaraldehyde (25 %) was added to the reaction mixture (with 1, 2, 3, 4 % end concentrations). pH was adjusted to 8 with triethanolamin buffer (pH 8). The reactants are incubated for 1h at room temperature at 250 rpm in a shaker incubator. The reaction was terminated by the addition of 1M Tris pH 8 (maximum 0.1 % (v/v)) followed by a 15 minutes incubation. Dialysis was performed in Cellusept dialysis membranes either at room temperature for 2 hours or at +4 °C in cold room overnight. Products were collected after dialysis.

Crosslinking under Vacuum with BSA:

Different parameters were used in crosslinking. 50 mg BSA was added to 1 ml of cellulase enzyme in 50 ml ddH₂O. The solution was dialyzed overnight against ddH₂O at 4 °C. The dialysis products were lyophilized and the samples were incubated under vacuum at 65 °C overnight (CLV-O/N) and for one hour, two hours (CLV-2H).

20 ml of the dialysis products were taken and 30 ml of ddH₂O was added and pH adjusted to 8 with triethanolamin. Glutaraldehyde with an end concentration of 2 % was added and incubated for 1 hour at 25 °C, 200 rpm. The reaction was quenched with 5 ml 1M pH 8 Tris and dialyzed overnight at 4 °C and lyophilized (CLV-Glu).

Crosslinked enzyme was prepared as in 3.1.3.1 with a minor difference. After quenching 50 mg BSA was added per 50 ml solution and lyophilization was performed then (CJB).

4.2.1.4 Lyophilization

Dialysis products were taken into Falcon tubes (~15 ml of liquid sample) and the samples were freeze-dried using liquid nitrogen and freeze-dried in lyophilizer.

4.2.1.5 SDS-PAGE analysis

12 % Separating, 5 % Stacking gels were prepared as in Appendix B.

4.2.1.6 Zymogram

12 % SDS-PAGE gel containing 0.1 % CMC in the separating gel was prepared and run following the procedure in Appendix B without performing the staining and destaining steps. The gel was washed three times (each 15 minutes) with 2.5 % TritonX-100 on a benchtop shaker. The gel was washed with 0.05 M pH 4.8 NaOAc buffer and then incubated in 0.05 M pH 4.8 NaOAc buffer for 1 hour in the benchtop shaker incubator at 55 °C. The gel was then incubated with Congo red solution (0.05 %) for 2 hours and incubated in 0.05 M pH 4.8 NaOAc buffer overnight to allow destaining.

4.2.1.7 Enzymatic Biofinishing of Viscose Fabrics

Biopolishing of viscose fabric with native and modified enzyme samples was performed in shaker incubators under constant shaking (200-400 rpm) at 45 and 55 °C, pH 4.8 in 0.05 M NaOAc buffer for 1 hour. Liquor ratio of 1:15 was used in all tests. 0.4 % (of the total liquor volume) native enzyme was used for the tests. Two different concentrations of the modified enzymes were used. One was the modified enzyme having equivalent protein concentration according to the Bradford Protein Assay (protein equivalent) and the other was fraction of the modified enzyme exhibiting the same activity as the native enzyme according to the Endoglucanase Activity Assay (activity equivalent).

4.2.1.8 SEM Characterization

SEM characterization of the enzyme treated, modified enzyme treated and untreated fabric and fibers was performed with Gemini Supra 35VP without coating and under low accelerating voltage (0.9 kV) and with a working distance of 4 mm using the Inlens detector .

4.2.1.9 Pilling Test

All the tests were performed in Ege University Textile Engineering Department. Martindale 2000 pilling machine was used at 2000 rpm. The reference photographs used were EMPA Standart SN 198525 K3. The photographs were evaluated according to AATCC (Association for American Textile Chemists and Colorists) standards by five referees with eye examination. All the values are the weighted averages of the five measurements. For pilling measurements, a five-point evaluation system is used. 1 indicates intense pilling and 5 indicates no pilling.

4.2.1.10 Bursting Strength Test

Bursting strength tests for enzyme treated and untreated viscose fabrics were performed in James H. Heal testing machine according to AATCC. Quadruple measurements were taken for each sample fabric and the end values were the weighted averages of those.

4.2.2 Immobilization of GOx, Desizing and Biobleaching

4.2.2.1 Immobilization Methods

4.2.2.1.1 Immobilization on Alumina

10 g of Al_2O_3 was incubated for 10 minutes in a 100 ml solution of 10 % APTS in 50 % ethanol in a shaker incubator at 100 rpm at 25 °C . The supernatant was discarded. The beads were washed four times with ddH₂O and the Alumina beads were dried and kept in a 60 °C oven for further use. 2 g of silanized Alumina beads were incubated overnight at different glutaraldehyde concentrations at 25 °C and 100 rpm. The beads were centrifuged and the supernatant was discarded and the beads were washed four times with ddH₂O. 0.25 g GOx in 50 ml ddH₂O was added onto the beads and they were incubated at 25 °C for 30 minutes at 300-400 rpm. The solution was centrifuged and the supernatants were taken into separate tubes for characterization of the unbound protein and the beads were washed four times with ddH₂O. All supernatants after washing step were kept in separate tubes.

4.2.2.1.2 Immobilization on Silica

1 g silica beads (63-200 mesh and 100 Å pore size) were first coated with 10 % APTS in 50 % ethanol for 10 minutes at 25 °C, 100 rpm and beads were incubated in 1, 2, 3 and 4 % glutaraldehyde containing solution for 30 minutes at 25 °C, 100 rpm. Because of red color formation this method was abandoned.

1 g Silica beads were silanized with 5 % 3-glycidoxypyltrimethoxy silane (GPTS or TMGS) in the presence of 77 % methanol, 3 % acetic acid and 15 % water at 25 °C overnight with constant shaking (250 rpm). Then the solution was centrifuged and the supernatant was discarded. The beads were dried and kept in a 60 °C oven for further use. Different concentrations of GOx were incubated with 0.2 g silanized beads with a total volume of 5 ml at 25 °C, 400 rpm for 30 minutes. The solutions were

centrifuged and the supernatants were taken into separate tubes for characterization of the unbound protein and the beads were washed four times with ddH₂O. All supernatants after washing step were kept in separate tubes.

4.2.2.1.3 Immobilization on CNBr activated Sepharose-4B

The immobilization on CNBr activated Sepharose-4B was performed exactly following the users manual supplied by Sigma Chemicals. Appendix C shows the users manual.

4.2.2.1.4 Crosslinked Enzyme Aggregates-CLEAs

Preliminary Studies:

3.3M, 2M and 1M Ammonium sulphate was prepared in 0.1M pH 7.3 Potassium phosphate buffer. 1 ml solutions of 2.5, 5, 10 mg/ml GOx were prepared in Potassium phosphate buffer and added dropwise to 9 ml of 1, 2, 3.3M Ammonium sulphate solution. 5 ml, 2.5 % glutaraldehyde with 1 % (v/v) phosphoric acid was prepared and the pH is adjusted to 7.3 with NaOH. Glutaraldehyde was added to the solution of enzymes and precipitants with an end concentration of 1 %. The solutions were incubated for 2.5 hours at 28 °C, 250 rpm. After the incubation 0.1M Sodium borohydride is added to convert imines to secondary amines and incubated for 10 minutes. 10 ml of phosphate buffer was added. Then the solutions were centrifuged and the precipitants were washed with phosphate buffer. Formed aggregates were resuspended in 1 ml phosphate buffer.

Other studies:

4M Ammonium sulphate was prepared in 0.1M pH 7.3 phosphate buffer. 25 mg/ml GOx was prepared in phosphate buffer and added dropwise to 9 ml of 4M Ammonium sulphate solution. Three different concentrations of glutaraldehyde were used (0.3, 0.5 and 0.7). Glutaraldehyde was prepared as in 3.2.1.5.1. The solutions were incubated for 2.5 hours at 28 °C, 250 rpm. After the incubation 0.1M Sodium borohydrite is added to convert primary imines to secondary amines and incubated for 10 minutes. 10 ml of phosphate buffer was added. Then the solutions were centrifuged and the precipitants were washed with phosphate buffer. Formed aggregates were resuspended in 1 ml phosphate buffer.

4.2.2.2 Determination of Immobilization yields

Immobilization yields were determined using Bradford protein assay. The first supernatants after incubation and the supernatants of the subsequent washing cycles were subjected to Bradford protein assay as well as the samples having the initial protein concentration. The immobilization yield was calculated as:

$$\% \text{ Yield} = (E_1 - E_2) * 100 / E_1$$

E_1 = Initial Enzyme Concentration

E_2 = Enzyme Concentration in the Supernatant

4.2.2.3 Activity Tests

For the activity determination preliminary studies were performed with Amplex Red Assay however the activity test were failed with the imobilized enzymes since the activities were so high even for 0.5 mg support that the peroxide production was beyond the limits of the assay. So KMnO_4 titration was used instead.

KMnO_4 Titration:

Activities were assigned according to hydrogen peroxide production of the enzymes from glucose. Titration was performed according to AATCC Test Method TM 102-1997 (See Appendix D).

Amplex Red Glucose/Glucose Oxidase Assay:

Amplex Red glucose/glucose oxidase assay was performed according to the manufacturer's instructions (See Appendix E).

4.2.2.4 Desizing of Cotton Fabric

Cotton fabrics were desized at a liquor ratio of 1:10 using 1 % (enzyme volume (ml) / g dry fabric weight) amyloglucosidase at 63 °C in 0.05M, 0.1M NaOAc, pH 4 in the hybridization oven or in the shaker incubator at 250 rpm.

4.2.2.5 Hydrogen peroxide production with GOx

H₂O₂ production against 50mM glucose at 55 °C and at pH 4 and 7 in 0.05M NaOAc with 0.1 % Rucogen for 1 hour was monitored to find the optimum conditions with different enzyme dosages. The trials against the previously desized cotton fabric were performed at 55 °C and at pH 4 and 7 in 0.05M, 0.1M NaOAc buffers for 1 hour. All the trials were performed using a shaker incubator at 250 rpm or a hybridization oven. In the trials with pH 7, the pH of the liquor was increased using 5M NaOH. The effects of aeration on the peroxide production was examined by performing the reaction in 250 ml Erlenmayer flasks and by introducing holes on the openings of the flasks capped with aluminium foil. Peroxide production was analyzed using KMnO₄ titration.

4.2.2.6 Biobleaching of cotton fabric

Biobleaching of the cotton fabric was carried on for 1 and 2 hours at 95 °C with constant rotation in the hybridization oven and for 3 hours at 80 °C in the shaker incubators with constant shaking. Residual peroxide in the bleaching bath was monitored using KMnO₄ titration. Two types of bleaching was performed. The conditions were:

Activator Bleaching:

1 % Prestogen SP (of fabric weight) was added and the pH of the liquor solution was adjusted to 7 with 5M NaOH. Drop by drop adjustment of the pH was necessary since the peroxide dissociates rapidly at high pH without stabilisers.

Alkaline Bleaching:

Peroxide stabilizer BTCA 0.1 % was added and the pH was adjusted to 11 with 5M NaOH.

4.2.2.7 Determination of Whiteness Index

Whiteness Index of the bleached fabrics were determined according to Stensby equation in Reflectance Grademacbeth Spectrophotometer under D65/10° light.

CHAPTER 5

RESULTS

5.1 Viscose Biopolishing

5.1.1 Optimization & Characterization of Crosslinking

5.1.1.1 Optimization of [glutaraldehyde]

Figure 5.1 indicates the effects of glutaraldehyde concentrations on the activities of the crosslinked Gempil 4L samples. According to the results, best activities were attained at 3 % glutaraldehyde concentration but the activities attained using 1 and 2 % glutaraldehyde were also very close to the one attained by 3 %. At 2 % [glutaraldehyde] and after 3 % there was a decrease in the activities.

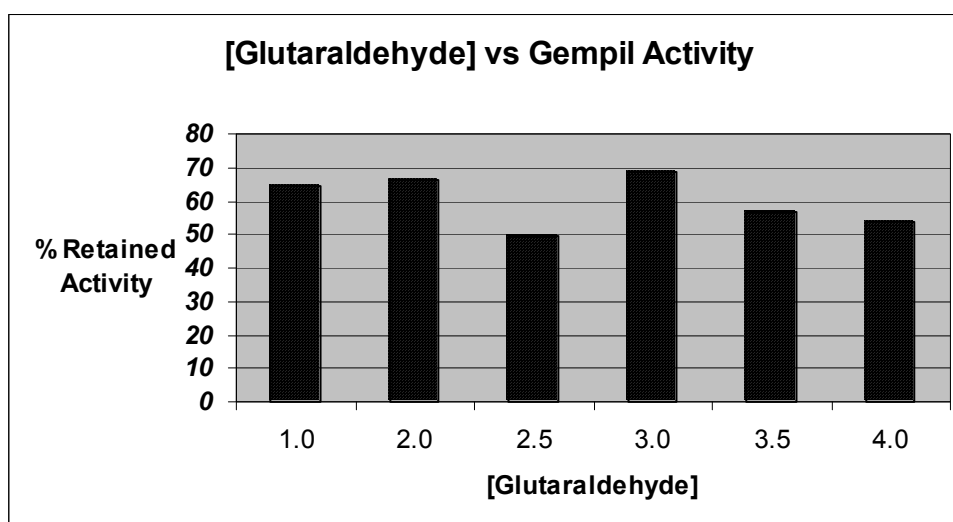


Figure 5.1: Effects of glutaraldehyde concentrations on the activities of the crosslinked Gempil 4L enzyme samples. Retained activities of the enzymes were calculated as follows: Retained activity= Activity of the modified enzyme*100/ Activity of the native enzyme having the same protein concentration.

5.1.1.2 SDS-PAGE Analysis

The extent of crosslinking of the commercial enzyme samples were analyzed using SDS-PAGE (12 %) analysis (Figure 5.12). The enzyme samples were loaded at equal protein concentrations into the gel. The protein bands of the modified samples correspond to the uncrosslinked portions. According to the results, G(+) samples seem to be crosslinked since the uncrosslinked portion visualized in the gel was smaller with respect to G(-) sample at the same protein concentration. The results were double checked. The CLV samples also appear to be crosslinked (Figure 5.12-a) with respect to G(-) samples in the gel in Figure 5.12-b. They were all at the same protein concentrations.

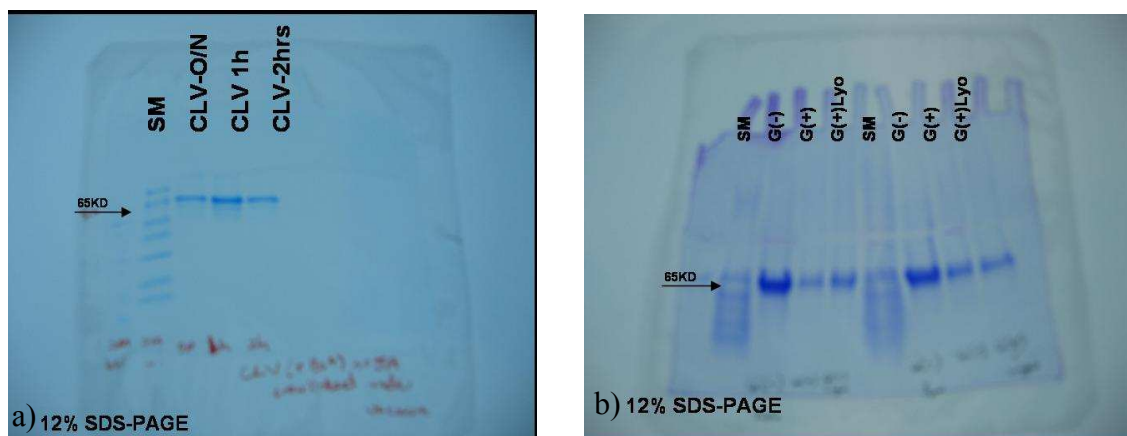


Figure 5.2: a) SDS-PAGE (12 %) analysis of the Gempil 4L samples crosslinked with BSA under vacuum for 1 hour, 2 hours, overnight at 65 °C. The 65 kDa band corresponds to BSA. b) SDS-PAGE (12 %) analysis of modified (2% [Glutaraldehyde]) and native Gempil 4L at the same protein concentrations.

5.1.1.3 Cellulase Activity

5.1.1.3.1 Temperature Activity Profile

Figure 5.3 shows the normalized activity results for native Gempil 4L and modified Gempil 4L at different temperatures. The best activities were at 55 °C and 65 °C for G(-) and G(+), respectively. It was found that the temperature optimum of the enzyme shifted to 65 °C upon crosslinking with 0.05 % glutaraldehyde. Normalized temperature activity profile of native and modified IndiAge 2XL was shown in Figure 5.4. Activity of the modified enzyme follows a rising trend upto 75 °C and after that the activity drops quickly and it seems to be more active compared to the native enzyme at temperatures over 55 °C. Temperature activity profile of native and modified Gempil 4L was shown in Figure 5.5.

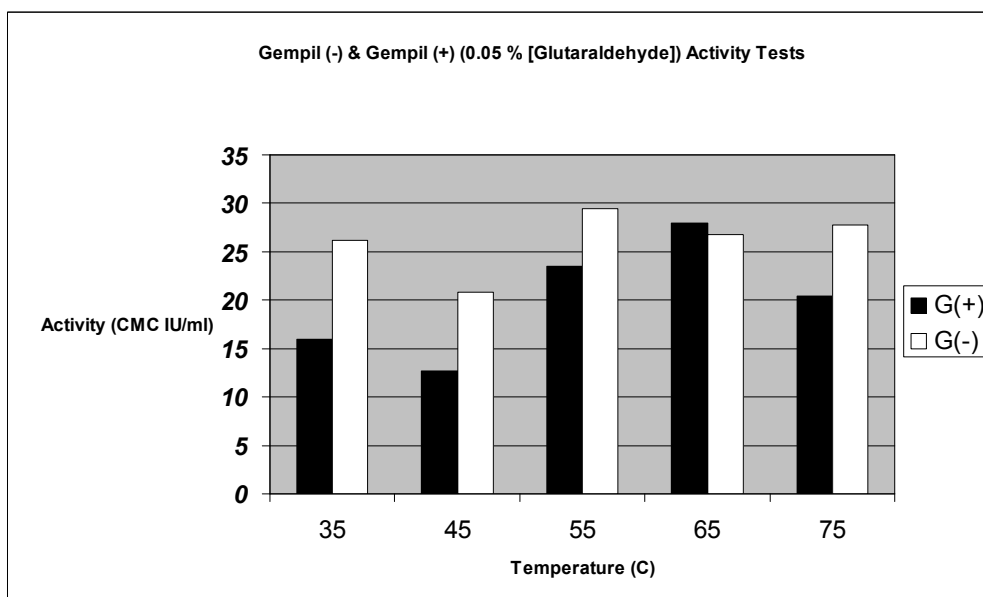


Figure 5.3: The normalized activity results for native Gempil 4L-Concentrated/ G(-) and modified Gempil 4L-Concentrated/ G(+) at different temperatures.

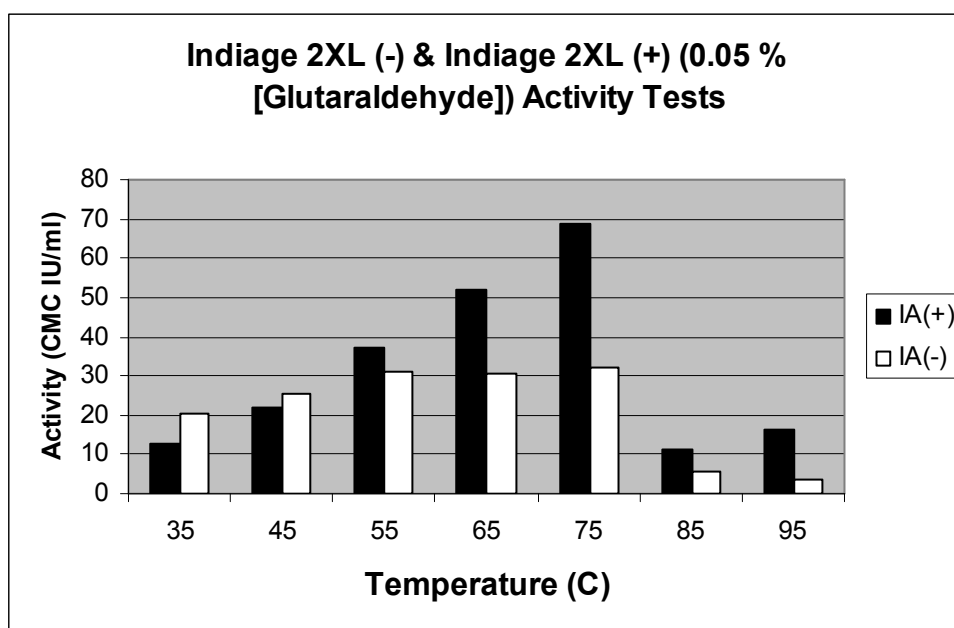


Figure 5.4: Normalized temperature activity profile for IA(-) and IA(+) at 0.05 % [glutaraldehyde].

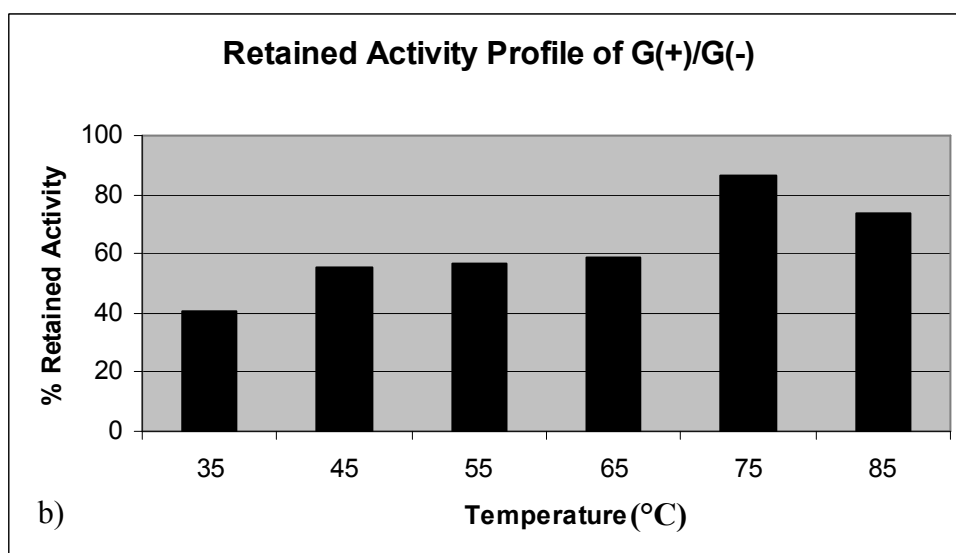
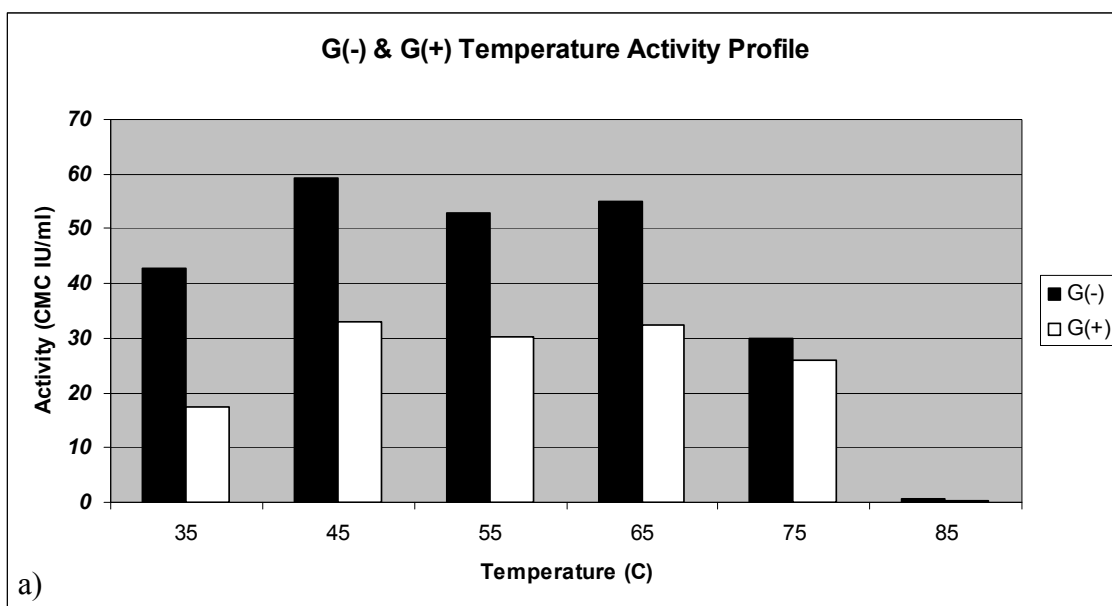


Figure 5.5: a) Temperature activity profile of the native Gempil 4L and crosslinked, lyophilized Gempil 4L at the same protein concentration and with 2 % [Glutaraldehyde]. The optimum range of activity of the enzyme was changed from 35-65 °C to 45-75 °C. b) % Retained activity of G(-) upon crosslinking.

The temperature activity profiles of native and modified Gempil 4L were indicated in Figure 5.6. The native Gempil 4L (G(-)) seems to function over a broad activity range (45-65 °C). Dialysis of the native Gempil 4L resulted in loss of enzyme activity due to the loss of a portion of the enzyme components (GL(3/100) DIAL). The temperature activity profile of the dialyzed enzyme was similar to G(-) but the activities were reduced. Addition of BSA to G(-) and dialysis of BSA added G(-), also resulted in loss of some activity. The temperature activity profile exhibited a peak at 55 °C. In case

of crosslinked Gempil 4L-Jeffamine networks (G(+)), the temperature activity range of the enzyme appears to be broadened (45-75 °C) with respect to G(-) and there was also some decrease in the activities at all temperatures except 75 °C compared to the free enzyme. On the other hand, the temperature activity profile of CLV-2h exhibited a peak at 55 °C and its profile was similar to the BSA added and dialyzed sample (G(-)+BSA). The temperature activity profile of overnight crosslinked sample, CLV-O/N appears to resemble the free enzyme's profile but with a reduced activity. The increase in the incubation times under vacuum caused a small decrease in the activities as expected since as the incubation time increases under vacuum the extent of crosslinking would increase and the activities were reduced as a result. In case of CLV-Glu, introduction of both vacuum and glutaraldehyde for crosslinking, reduced the activity of the enzyme nearly three-folds at 55 °C however the modified enzyme preserved its broad range of activity. CJB samples exhibited the lowest activity for all temperatures. CLV-2h and G(+) were selected for use for the other experiments.

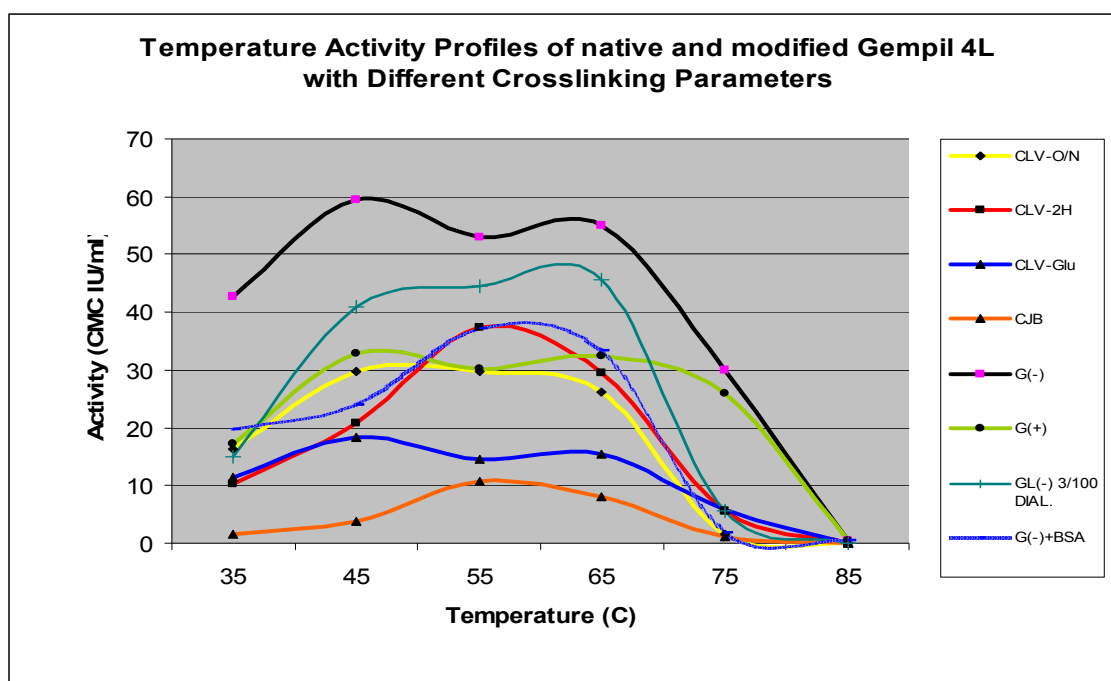


Figure 5.6: Temperature activity profile of native and crosslinked Gempil4L under different conditions. CLV-O/N: Gempil 4L samples crosslinked with BSA under vacuum overnight at 65 °C. CLV-2H: Gempil 4L samples crosslinked with BSA under vacuum for two hours at 65 °C. CLV-Glu: Gempil 4L samples crosslinked with BSA under vacuum with the addition of glutaraldehyde. CJB: Gempil 4L samples crosslinked with Jeffamine with the addition of glutaraldehyde and BSA was added after quenching. G(-): Native Gempil 4L samples. G(+): Crosslinked Gempil 4L with 2 % glutaraldehyde. GL(-)3/100: Gempil 4L sample 3/100 dilution from the original stock and dialyzed. G(-)+BSA: BSA added to 3/100 dilution of G(-) and dialyzed.

5.1.1.3.2 pH Activity Profile

Optimum pH for both native and crosslinked enzymes was found to be pH 5. The activity test failed at pH 3 and 4 by the formation of an unknown precipitate. The enzymes exhibit very low activity above pH 7 (Figure 5.7).

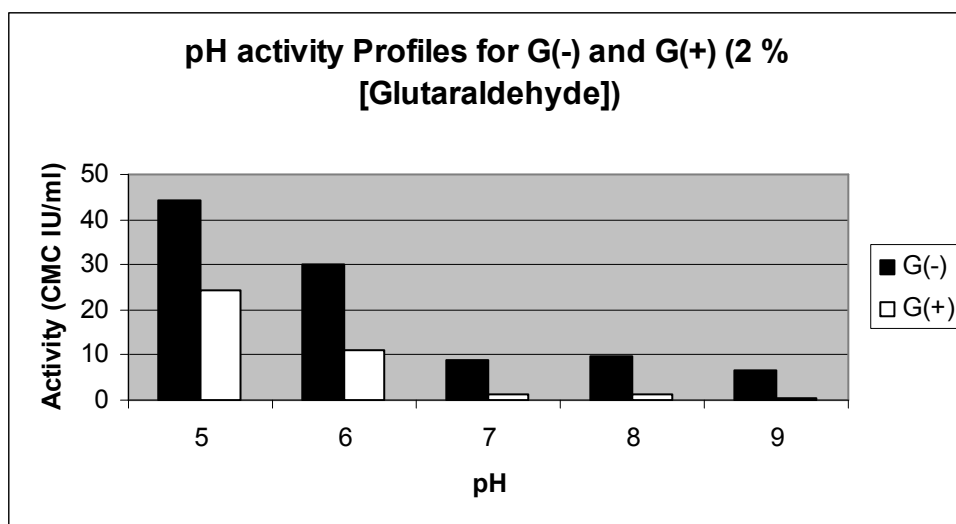


Figure 5.7: pH activity profiles for G(-) and G(+) crosslinked in the presence of 2 % [glutaraldehyde].

5.1.1.3.3 Stability

Preliminary stability studies were performed on native and the crosslinked enzyme G(-) and G(+) at 65 °C for 4 hours against 1 % CMC. The results indicate that stability of the native enzyme was more than stability of the crosslinked enzyme for the first four hours at 65 °C (Figure 5.8). Crosslinked cellulase sample under vacuum for 2 hours (CLV-2h) appeared to be more stable than the native enzyme at 65 °C (Figure 5.9).

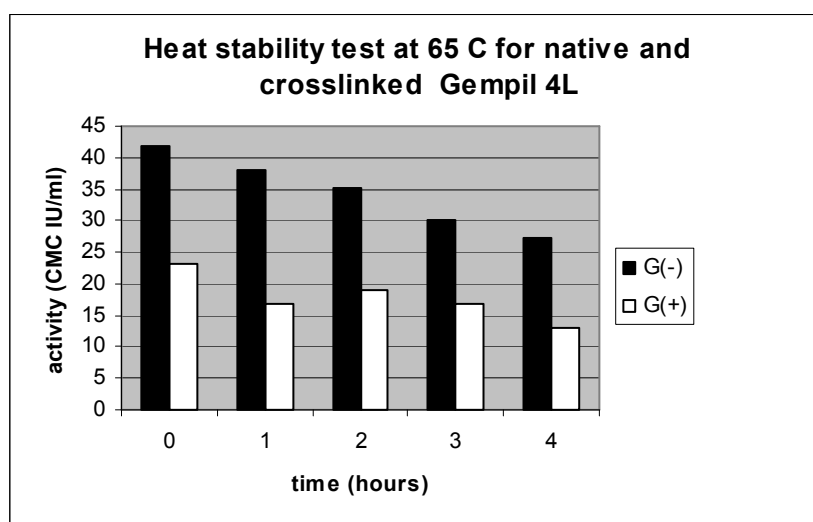


Figure 5.8: Heat stability test performed at 65 °C for four hours with $[G(-)] = [G(+)]$. 0.32 mg/ ml of G(+) and 1/100 dilution of G(-) stock were used in the experiment.

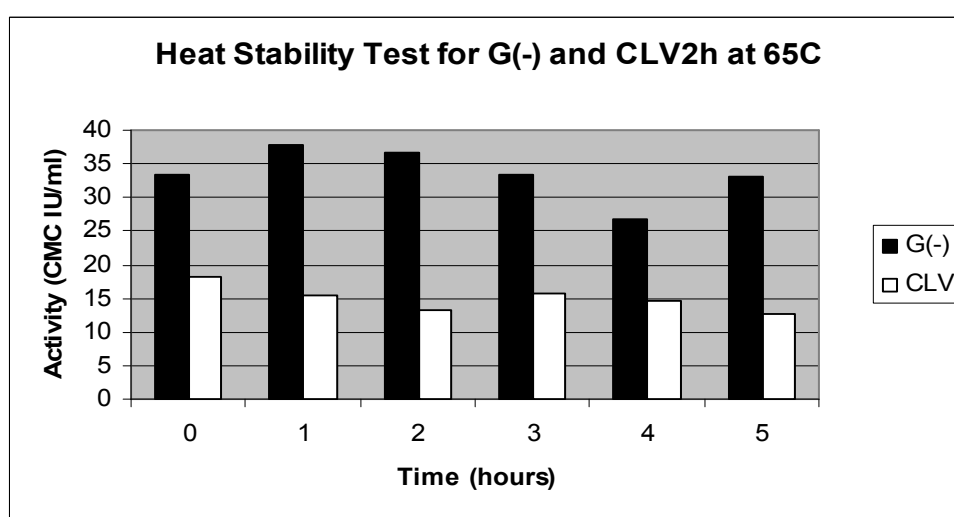


Figure 5.9: Heat stability test performed at 65 °C for five hours with $[G(-)] = [CLV2h]$. 1.5 mg/ ml of CLV2h and 1/100 dilution of G(-) stock were used in the experiment.

Reducing sugar production was the most for 24h incubation of the viscose with native cellulases (G(-)) and the most for 96 h incubation of the viscose with crosslinked cellulases (CLV-2h) (Figure 5.10).

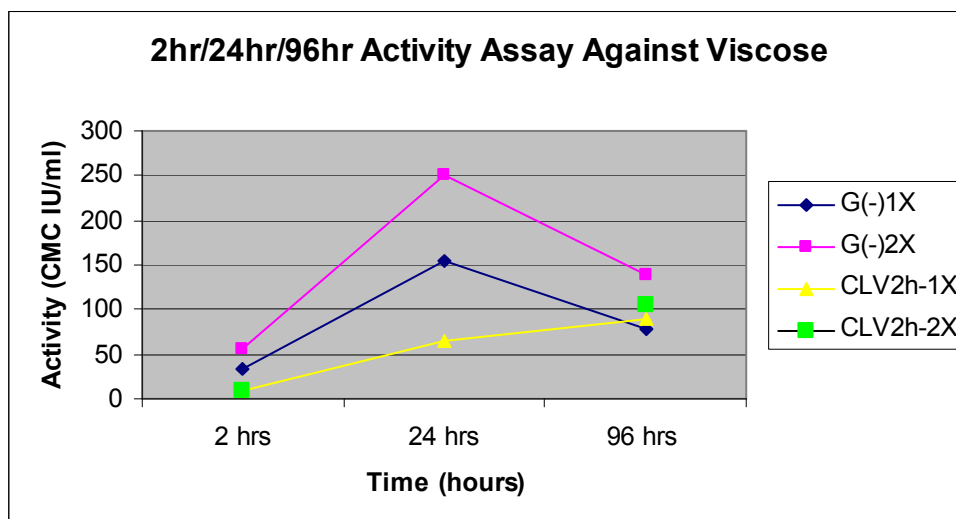


Figure 5.10: Normalized activities against viscose fabric after 2, 24 and 96 hours incubation. G(-)1X has the same protein concentration with CLV2h-1X and G(-)2X has the same protein concentration with CLV2h-2X.

5.1.1.3.4 Zymogram

Zymogram analysis was performed to analyze the activities of crosslinked and native enzymes (Figure 5.11). All enzymes crosslinked and native have the same protein bands and these bands are the ones having the cellulolytic activity. All enzymes were loaded at equal protein concentrations so the band intensities correspond to the cellulolytic activities. Only in case of the crosslinked enzymes the portion that is seen on the gel was the portion that is not crosslinked. The crosslinked portion could not enter the gel. Activities of Gempil 4L and Eonase CE were the highest and activity of the crosslinked enzyme was the lowest (uncrosslinked portion).

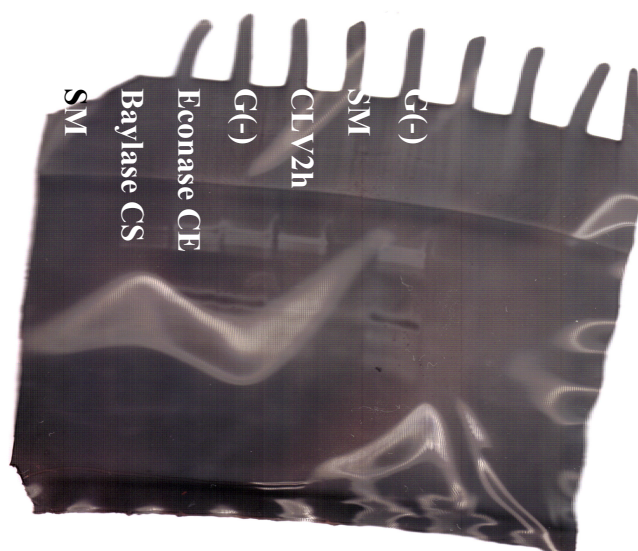


Figure 5.11: Zymogram of native (G(-)) and modified Gempil 4L (CLV2h) and other commercial enzyme preparations, Baylase CS and Econase CE, all at the same protein concentration in a 12 % SDS-PA gel prepared having 0.1 % CMC.

5.1.1.4 Fabric Trials

5.1.1.4.1 SEM Analysis of Fabric Surface and Fibers

Figure 5.12 indicates the SEM analysis of viscose fabric treated with crosslinked and native cellulases. The results indicated no visual changes on the fabric surface upon enzymatic treatment under 185X magnification. However, the fiber surface appeared to be polished with native enzyme but not with the crosslinked enzyme (Figure 5.12b). There was one exception to this. Upon prolonged incubation (3 hours) crosslinked cellulase also caused surface polishing (Figure 8.2, Appendix D). It was also found that use of two commercial lipases had no effect on the fiber and fabric structure microscopically (Figure 8.3, Appendix D).

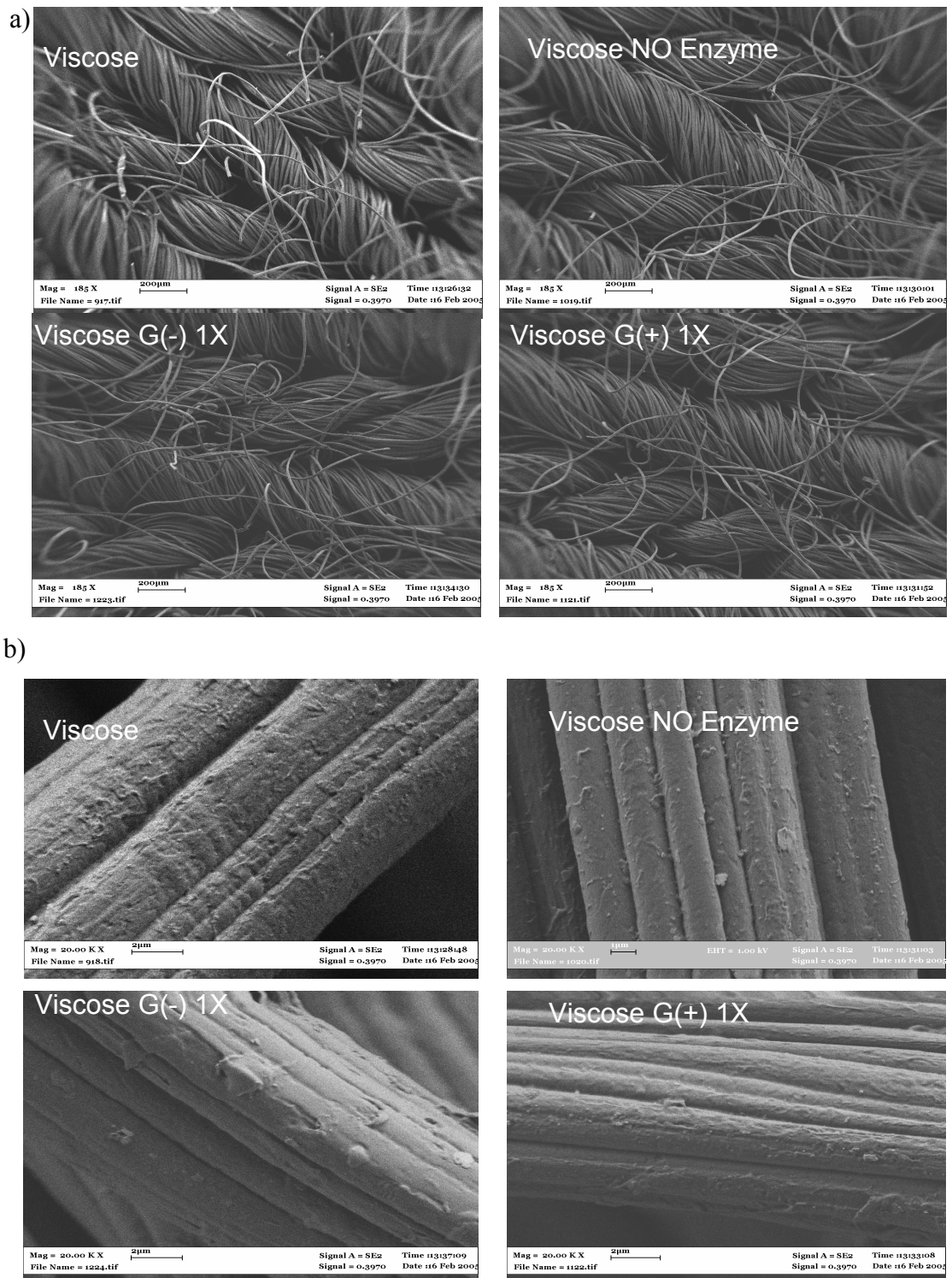


Figure 5.12: SEM pictures of untreated viscose fabric, viscose fabric incubated in 0.05M pH 4.8 NaOAc buffer, viscose fabric treated with native enzyme (G(-)) and crosslinked enzyme(G(+)) having the same protein concentrations. Treatment conditions: 1hour treatment at 400 rpm L.R.=1:15 at 55 °C) under a)185X magnification b) 20000X magnification.

5.1.1.4.2 Effects of RPM and Temperature

The strength of the fabric was best at 100 rpm at both 45 and 55 °C upon enzymatic treatment with native Gempil 4L (Figure 5.13). Release of reducing sugars appeared to peak at 200 rpm at both 45 and 55 °C but the highest peak occurred at 55 °C (Figure 5.14).

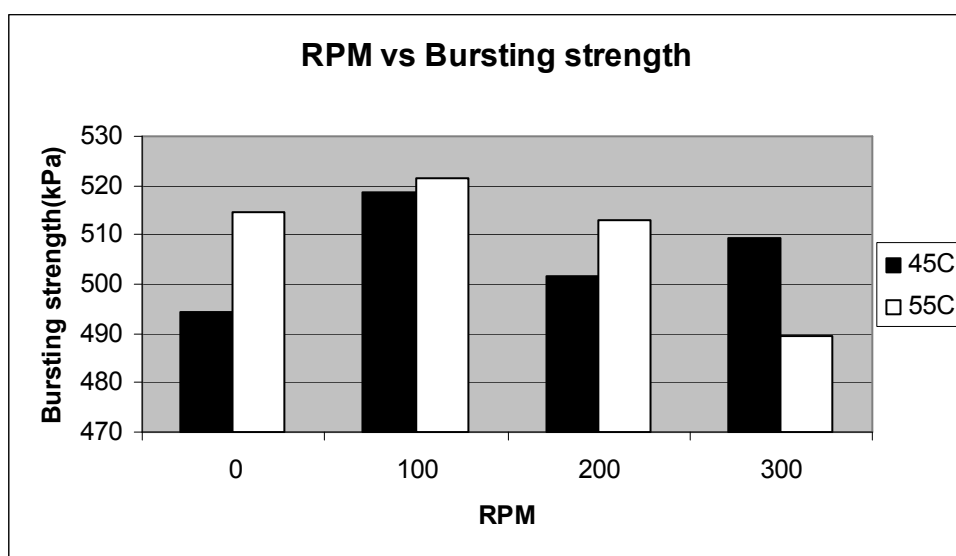


Figure 5.13: Effects of changing RPM on bursting strength of the viscose fabric at two different treatment temperatures. G(-) was used in the treatments.

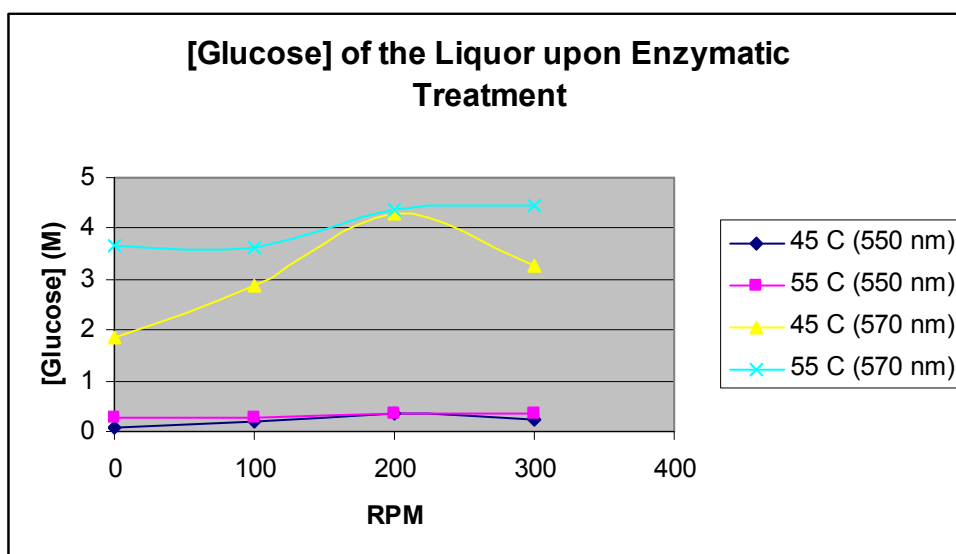


Figure 5.14: Reducing sugar production of viscose treated with native Gempil 4L at 45 °C and 55 °C monitored at 550 and 570 nm at different RPMs.

5.1.1.4.3 Effects of Incubation Time

The mechanical strength of the viscose fabric seems to be decreased with the increase in incubation time, only with the exception of five hours incubation time for both native and modified enzyme treatments. There was an increase in bursting strengths for five hours incubation in both samples. Incubation time seems to be a very important parameter in determining the degree of loss of fabric strength. One or two hours incubation time appears to be appropriate for the enzymatic process (Figure 5.15).

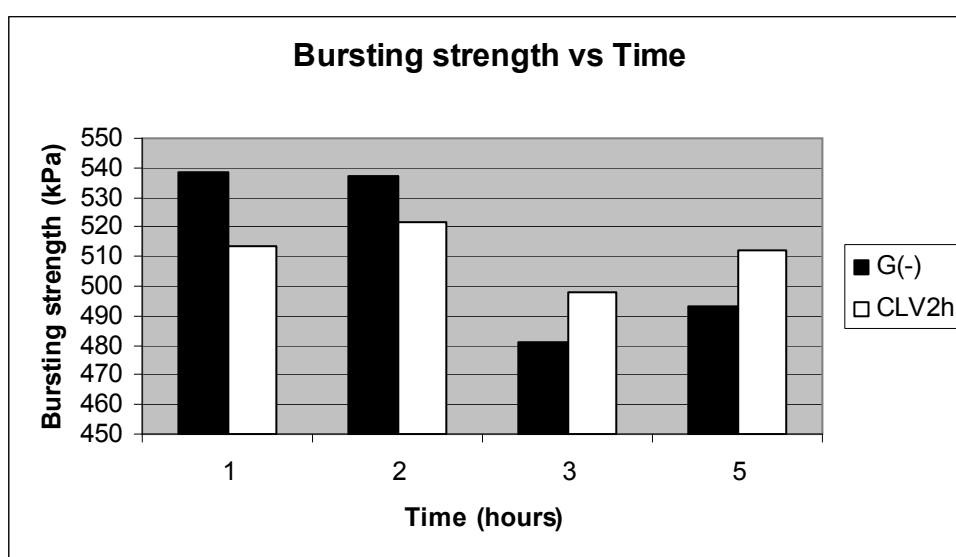


Figure 5.15: Effects of incubation time on mechanical strength of the viscose fabric upon G(-) and CLV2h treatment.

5.1.1.5 Effects of Surfactants

Untreated fabric was examined under 50X magnification and examination revealed the fibrils protruding from the surface of the fabric (Figure 5.16).

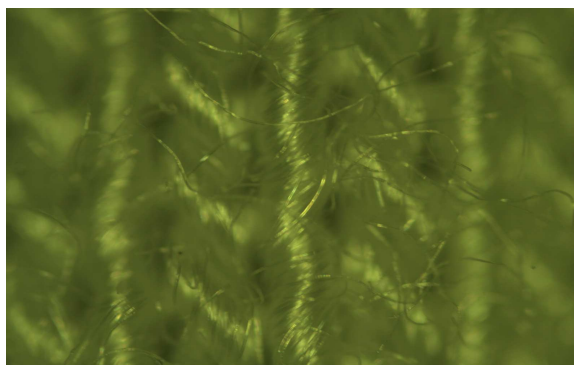


Figure 5.16: Untreated viscose fabric visualized under 50X magnification.

5.1.1.5.1 TritonX-100

Enzymatic treatment in the presence of 0.05 % TritonX-100 did not cause visible changes on the surface appearance of the viscose fabrics for both G(-) and G(+) treatments at 50X magnification compared to the untreated fabric.

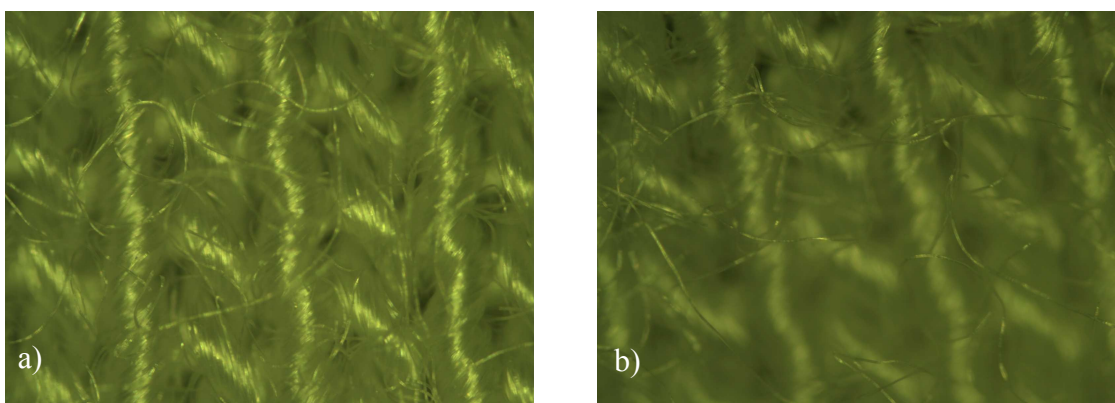


Figure 5.17: a) Effects of 0.05 % TritonX-100 and native Gempil 4L on the surface properties of viscose fabric. b) Effects of 0.05 % TritonX-100 and modified Gempil 4L on the surface properties of viscose fabric. Examined under 50X magnification.

5.1.1.5.2 Tween 20

Enzymatic treatment with 0.05 % Tween 20 did not cause visible changes on the surface appearance of the viscose fabrics for both G(-) and G(+) treatments at 50X magnification with respect to the untreated fabric.

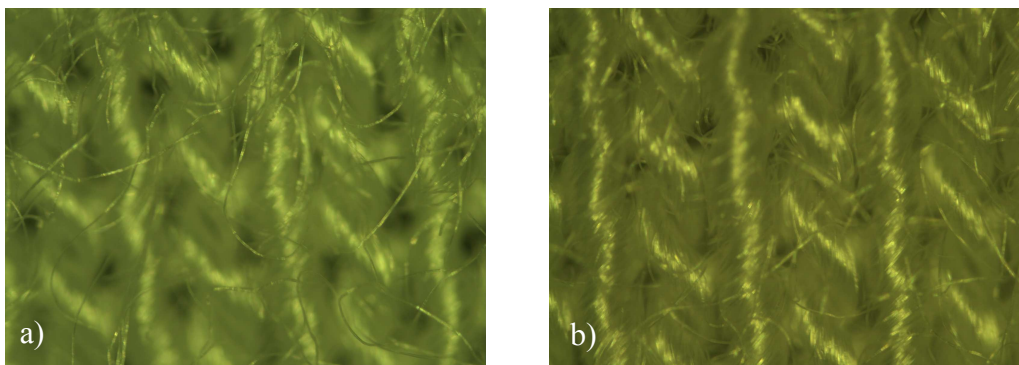


Figure 5.18: a) Effects of 0.05 % Tween 20 and native Gempil 4L on the surface properties of viscose fabric. b) Effects of 0.05 % Tween 20 and modified Gempil 4L on the surface properties of viscose fabric. Examined under 50X magnification.

5.1.1.5.3 Tween 80

Enzymatic treatment in the presence of 0.05 % Tween 80 did not affect the surface appearance of the viscose fabrics for both G(-) and G(+) treatments at 50X magnification with respect to the untreated fabric.

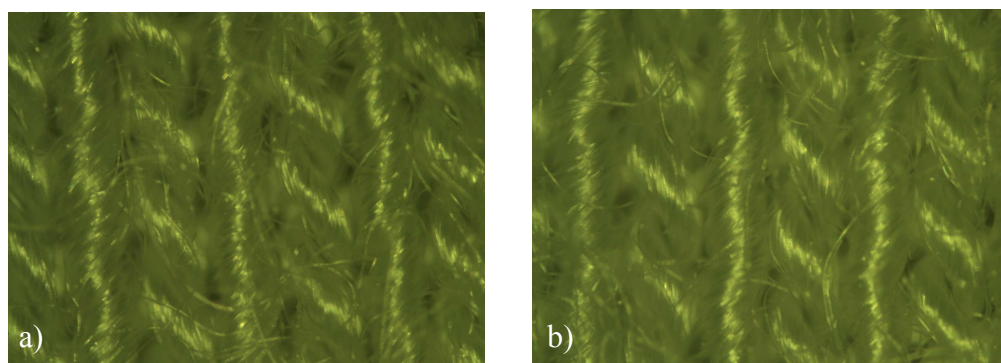


Figure 5.19: a) Effects of 0.05 % Tween 80 and native Gempil 4L on the surface properties of viscose fabric. b) Effects of 0.05 % Tween 80 and modified Gempil 4L on the surface properties of viscose fabric. Examined under 50X magnification.

5.1.1.6 Effects of Different Enzymes and Pretreatments on Pillings and Bursting Strength

Effects of different pre and after treatments on the surface and tensile properties of the fabrics were analyzed. Lipase pretreatment reduced the strength of the fabric without changing the pillings whereas lipase after treatment prevented the loss of fabric

strength caused by native cellulases, again without changing the pillings. Crosslinked enzyme-BSA networks (CLV-2h) were found to prevent the loss of fabric strength most. 10 % ethylene glycol treatment further improved its effect even at lower enzyme concentrations. 5 % ethylene glycol treatment did not improve the effect of the native enzyme. 0.25-0.5 unit differences in pilling evaluations were considered to be within the inspection error limits. All of the treatments positively or negatively effect the enzymatic action and as a result the bursting strengths but none of them had an effect on the pillings (Table 5.1).

Table 5.1: Effects of different types of treatments and pretreatments on pillings and fabric strength. EG= Ethylene Glycol, Lipase from Hog. Econase CE is a commercial cellulase, hemicellulase, xylanase enzyme mixture.

Treatment Type	Bursting Strength (KPa)	Pilling (Stensby Index)
G(-) treatment	430.8	2
G(-) pretreatment+ Lipase treatment	445.7	2
Lipase pretreatment+ G(-) treatment	422.3	2
10 % EG pretreatment+ G(-) treatment	435.2	1.75
CLV-2h treatment	495.4	2
10 % EG pretreatment+ CLV-2h (60 % activity) treatment	499.5	1,75-2
1 hours 5 % EG Treatment afterwashing with ddH ₂ O	536.9	-
Ethylene glycol %5 + Gempil (-) 55 °C 100 rpm 1 hrs	428.3	1.75
Econase CE 1h 55 °C 100 rpm	332	2

5.2.1 Immobilization of GOx

5.2.1.1 Al₂O₃-GOx conjugates

5.2.1.1.1 Immobilization Yields

Best immobilization yields were attained at 1400 rpm when 3 mg/ml GOx was immobilized on alumina beads. The yields were 55 % at 200 rpm when 5 mg/ml enzyme was immobilized. Increasing the mechanical agitation increased the yield to 73.6 % (Figure 5.20).

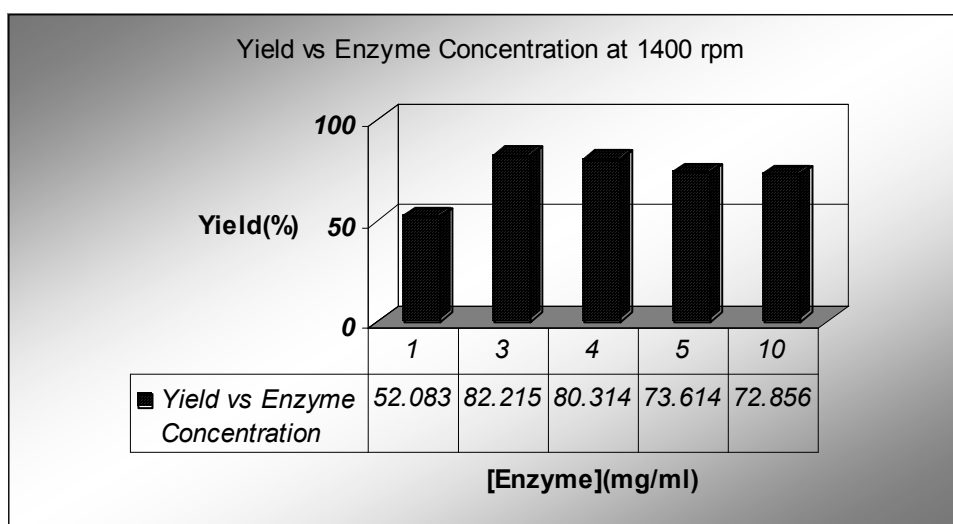


Figure 5.20: Effects of enzyme concentration on immobilization yields at 1400 rpm.

5.2.1.1.2 Activity Assays

Amplex Red Assay did not work due to the high activity of the IGOx.even for 0.5 mg and 1 mg supports.

5.2.1.2 Silica-GOx conjugates

5.2.1.2.1 Immobilization Yields and Activities

Increasing the native enzyme amount on silica support did not lead to an increase in H_2O_2 generation as it appears in G and H trials (Table 5.2). Increasing the amount of immobilized enzyme did not improved the H_2O_2 generation as it can be seen from A/B/C/D/E trials. It was expected to see a rising trend in peroxide generation as the native enzyme/ support ratio increased and to reach a maximum. Trial F was the optimized one for 0.05 gr silica with maximum H_2O_2 generation with 100 % immobilization yield and best activity was attained by sample F even 0.05 g of silica was used.

Table 5.2: Effects of different enzyme loadings on the immobilization yield on silica and H_2O_2 production, a comparison of native and modified enzymes at the same protein concentrations (activities against 50mM glucose at pH 7, 55 °C for 1 hour).

Sample	GOx/g support (mg)	[Enzyme] (mg/ml)	Yield	Silica (g)	[H ₂ O ₂] (mg/L) IGOx	[H ₂ O ₂] (mg/L) NGOx
A	1	0.04	100 %	0.25	23.8	102
B	2.5	0.1	100 %	0.25	27.2	108.8
C	5	0.2	100 %	0.21	105	153
D	10	0.4	100 %	0.23	90	136
E	20	0.8	100 %	0.25	137	277
F	30	1.2	100 %	0.05	99.7	183.6
G	40	1.6	71 %	0.05	114	183.6
H	50	2	84 %	0.05	112	183.6

Figure 5.21 shows the temperature activity profiles of selected enzyme samples from the previous table, IGOx and their equivalent GOx against 50mM glucose at pH 7 and at 55 °C for 1 hour. F, G, H denote IGOx having 30, 28, 42 mg GOx / g silica and F', G', H' denotes their corresponding NGOx. Best activities were at 25 °C for all enzymes except sample F.

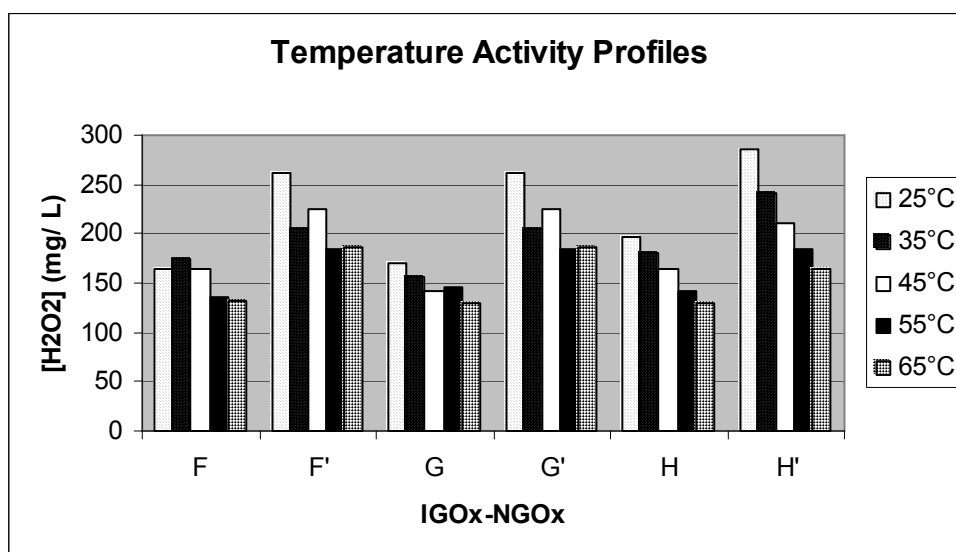


Figure 5.21: The temperature activity profiles of selected enzyme samples F,G and H (IGOx) and their equivalent NGOx against 50mM glucose at pH 7 and at 55 °C for 1 hour. F, G, H denote IGOx having 30, 28, 42 mg GOx / g silica and F', G', H' denotes their corresponding NGOx.

5.2.1.2.2 Fabric Trials

Table 5.3 indicates H_2O_2 production by native GOx (NGOx) and $[H_2O_2]$ before and after bleaching at different temperatures and two different pHs. According to these results the whiteness index was found to be best at 55 °C for the native enzyme and also for the immobilized enzyme. H_2O_2 production was best at 55 °C and at pH 7 for the native enzyme but the whiteness index values did not correlated with that. % utilization of peroxide by the fabric was also the most for the trials at 55 °C. However, amount of peroxide did not cause drastic/ incredible increases in the whiteness indexes. The whiteness indexes for the fabrics treated with GOx immobilized on silica (IGOx) were relatively low with respect to the NGOx treated fabrics. The whiteness index (WI) for the untreated fabric was 52.

Table 5.3: H_2O_2 production by native GOx (NGOx) and $[\text{H}_2\text{O}_2]$ before and after bleaching at different temperatures and two different pHs. Whiteness indexes of fabrics treated with NGOx and IGOx (Untreated fabric WI= 52). Bleachings were performed in hybridization oven or in shaker incubators in the presence of activators at pH 7.

	[H ₂ O ₂] (mg/L) NGOx		% H ₂ O ₂ utilization by fabric		Whiteness Index NGOx		Whiteness Index IGOx	
	Before Bleaching							
Temperature	pH 4	pH 7	pH 4	pH 7	pH 4	pH 7	pH 4	pH 7
25 °C	694	728	2	15	61	61	55	56
35 °C	629	639	20	12	61	61	56	56
45 °C	690	724	15	4	61	60	56	57
55 °C	843	877	29	23	63	62	57	57

Introduction of mechanical action with glass beads definitely increased the H_2O_2 production and aerated samples produced more H_2O_2 and the whiteness values were better. Bleaching performed at pH 6.5 also appears to cause a reduction in the whiteness levels (Table 5.4). Use of glass beads also increased the H_2O_2 production and whiteness index of the fabrics and use of fabric bags reduced the H_2O_2 production nearly to its half and on the other hand the whiteness index of the fabrics were reduced only 1 unit for IGOx (Sample F) (Table 5.5). In some of the samples it was observed that there is an increase in the peroxide concentration contrary to the expected results since fabrics use peroxide for bleaching. This may be explained by the fact that the enzyme is still active during bleaching even at 80 °C.

Table 5.4: Effects of glass beads, pH and aeration on H₂O₂ production and whiteness of the fabrics. Aeration was provided with Erlenmayer flasks and caps of the Falcon tubes were closed.

Sample	Glass Bead	Peroxide before bleaching	Bleaching Ph	Whiteness Index	Bleaching Conditions
1.5 mg NGOx	none	663	7	65	Aeration
	3.5 g	680	7.33	66	
	5 g	744.6	7.27	63	
	none	255	~6.5	57	Without Aeration
	1 g	255	~6.5	57	
	3.5 g	275.4	~6.5	57	
0.05 g IGOx	3.5 g	204	~6.5	57	

Table 5.5: The effects of glass beads and trapping the enzymes in fabric bags on H₂O₂ generation and fabric whiteness. Sample F was used in the trials (Activator bleaching). AD: After desizing step BB: Before bleaching step.

	Fabric weight	Bead weight	[H ₂ O ₂] (mg/L)		Whiteness Index
			Before Bleaching	After Bleaching	
No bags	3.23	5 g (AD)	289	299	59
	3.06	5 g (AD)	303	384	59
	3.22	5 g (BB)	309	258	59
	3.03	5 g (BB)	343	320	58
In bags	2.85	5 g (AD)	187	146	58
	2.94	5 g (AD)	153	163	58
	2.99	No Beads	160	156	57
	3.54	No Beads	136	163	57

Multiple use of the enzyme resulted in a reduction in H₂O₂ production and especially for samples B, C and D reusing after fifth cycle is not recommended.

(Figure 8.4 , Appendix D). The percent activity recovery after first use seems to increase with the increase in enzyme loadings. Multiple use of the enzyme (Sample F) resulted in a reduction in H_2O_2 production and also a 3 unit decrease was observed in the whiteness of the fabric after second use.

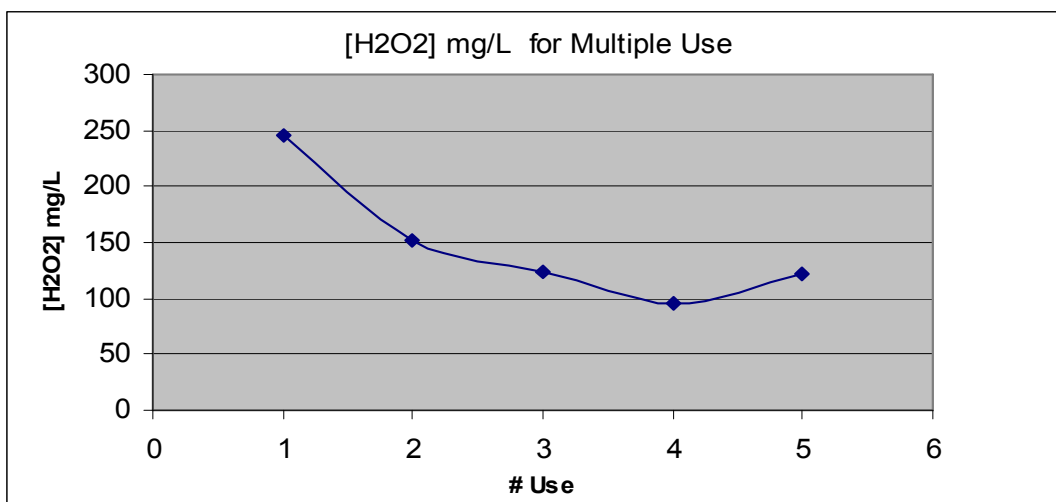


Figure 5.22: Multiple use of immobilized GOx

5.2.2 CNBr activated Sepharose 4B-GOx Conjugates

5.2.2.1 Immobilization Yields

1.8 mg GOx enzyme was immobilized on 0.1 g of CNBr activated Sepharose 4B and the immobilization yield was found to be 65.8 %. 0.1 g support contains 1.17 mg GOx.

5.2.2.2 Fabric Trials

Trials performed on desized fabric revealed that mechanical agitation created by glass beads increases peroxide production. Another interesting result is that 0.1 g Sepharose 4B has 1.17 mg GOx immobilized on it and immobilized enzyme appears to be more active than the silica-GOx conjugates although the whiteness levels reached with glass beads do not seem to be better than the silica-GOx conjugates.

Table 5.6: H₂O₂ production by immobilized GOx on CNBr activated Sepharose 4B and whiteness indexes of the treated fabrics.

Sample Enzyme	Fabric Weight	Type of Bleaching	[H ₂ O ₂] (mg/L) at pH 7		Whiteness Index
			Before	After	
0.1 g Sepharose 4B	3.57 g	Activator	289	221	-
	3.52 g		238	258.4	-
	3.48 g		289	255	-
0.1 g Sepharose 4B + 5 g glass beads	3.52 g		316.2	255	57
0.1 g Sepharose 4B + 3.5 g glass beads	3.53 g		302.6	278.8	57

5.2.3 Crosslinked Enzyme Aggregates (CLEA)

CLEAs of GOx were prepared with 1 % glutaraldehyde at different enzyme concentrations. It was shown that peroxide production and the retained activity % was increased upon increasing the enzyme concentration (Table 5.7). Moreover, increasing the enzyme concentration do not cause a linear change in the peroxide production the curve follows a parabolic trend in case of CLEAs and a hyperbolic trend in case of the native enzyme (Figure 5.23).

Table 5.7: Effect of CLEAs and enzyme concentration on H₂O₂ production and retained activities against 0.2 M glucose.

Enzyme	Dosage	[H ₂ O ₂] (mg/L)	Yield
NGOD	2.5 mg	571.2	45
CLEA		255	
NGOD	5 mg	649.4	50
CLEA		323	
NGOD	10 mg	717.4	60
CLEA		431.8	
NGOD	25 mg	856.8	51
CLEA		435.2	

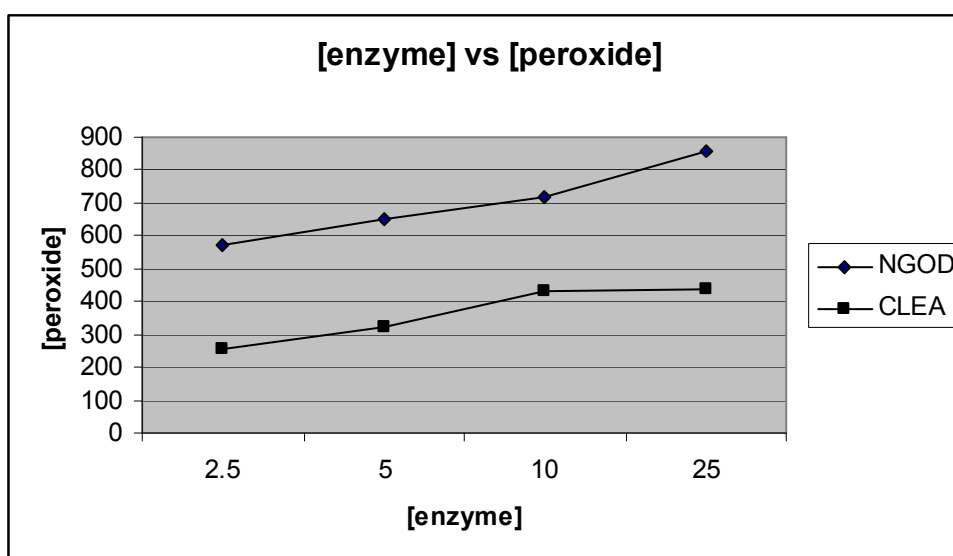


Figure 5.23: Effect of [enzyme] on [H₂O₂] in native GOx and CLEAs against 0.2 M glucose.

H₂O₂ production appears to be decreased with the increase in [glutaraldehyde] (Table 5.8) when 25 mg of GOx was used for precipitation. Best activities were attained at 0.3 % [glutaraldehyde]. Moreover, the activity retained with respect to the native GOx at the same concentration was 82 %. This value was the highest activity reached among all immobilization methods used.

Table 5.8: Effects of glutaraldehyde concentration on [H₂O₂] at 55 °C pH 7 against 0.05 M glucose in 0.1M NaOAc.

CLEA 25 mg	[H ₂ O ₂]
0.3 % [Glutaraldehyde]	703.8
0.5 % [Glutaraldehyde]	574.6
0.7 % [Glutaraldehyde]	554.2
1 % [Glutaraldehyde]	435.2

Table 5.9 indicates the repeated use of CLEAs prepared from 10 mg of GOx at 1 % glutaraldehyde concentration for three cycles against 0.2M glucose in 0.1M pH 7 NaOAc buffer at 55 °C . The activity of the enzymes were decreased upon repeated use.

Table 5.9: Repeated use of CLEAs (10 mg GOx and 1 % [glutaraldehyde]) and resulting H₂O₂ production.

	[H ₂ O ₂]		
	Trial 1	Trial 2	Trial 3
CLEA 10 mg	431.8	306	227.8

5.2.4 Optimization of Cotton Desizing and Biobleaching

Pretreatment of the fabric with a commercial cellulase, hemicellulase and xylanase formulation appears to cause a reduction in the whiteness index of the cotton fabrics. Increasing the concentration of Econase CE caused an increase in peroxide concentration but a decrease in whiteness index of the fabric (Table 5.10). Moreover, alkaline bleaching caused a 3 to 4 units increase in the whiteness index of the cotton fabrics as expected.

Table 5.10: Effects of treatment with Econase CE and different types of bleaching on peroxide production by NGOx and whiteness index of the fabrics. AG denotes Amyloglucosidase. NGOx was used for peroxide production.

Desizing	Fabric Weight(g)	Type of Bleaching	[H ₂ O ₂] (mg/L)	Whiteness Index
Econase 0.33 % + AG	3	Activator	578	59
Econase 0.17 % + AG	3		527	60
AG	3.03		561	62
AG	3.37	Alkaline	646	65
AG	3.14		625.6	66
AG	2.92		557.6	65

CHAPTER 6

DISCUSSION

6.1 Viscose Biopolishing with Native and Crosslinked Cellulases

6.1.1 Problems in Viscose Biopolishing: Overview

Formation of pills on the surface of the fabric gives fabrics an aesthetic appearance. Application of cellulases for the removal of the pills on the surface is widely used in the industrial processes. The enzymatic process is very convenient for the cotton fabrics but results in the loss of tensile strength in viscose fabrics. Another problem arises from the fact that most of the commercial cellulases are unable to increase pilling values of the viscose as other fabrics.

Viscose knitted fabrics are more prone to pilling than any of the fabrics because of their structure and fiber properties. The outer shell of the viscose fiber consists of amorphous cellulose separated by smaller ordered crystalline regions. Since the amorphous regions are more, the viscose fibers are more prone to attack by cellulases. Degradation of amorphous regions would provide easy access of the enzymes to the ordered crystalline regions. Since the crystalline regions are mainly responsible for the tensile strength along the fiber axis, the tensile strength of the fabric drops upon cellulase action because of the degradation of this highly ordered crystalline regions. Crosslinked Enzyme-Jeffamine or Enzyme-BSA networks can be used to alleviate the problem of lost tensile strength.

6.1.2 Effects of Crosslinking, pH and Temperature

The crosslinked Enzyme-Jeffamine networks (G(+)) obviously caused a lesser decrease in bursting strength percentages with respect to the free enzyme (G(-)) and resulted in a 0,25 unit increase in pilling values on a 5 unit scale (5-No pilling, 1-Strong Pilling). Formation of such an enzyme matrix may have restricted the enzyme onto the fabric surface as indicated by the SEM pictures of the fibers. Doubling the process times caused a slight decrease in the bursting strength of the fabrics. The near native-like rate measurements using Carboxy methyl cellulose (CMC) substrate indicated that the enzyme-polymer networks formed at lower glutaraldehyde concentrations might not be sterically hindered as the enzyme-polymer networks formed at higher glutaraldehyde concentrations and the substrate could potentially access the active center of the previous networks easier than the latter. Moreover the enzyme polymer networks crosslinked with 0,05 % glutaraldehyde displayed a slightly improved apparent activity and a ten-degree rise of temperature stability. The pH, temperature activity profiles of the latter networks indicated that the optimum conditions for the enzyme system did not change much upon crosslinking. Crosslinked enzyme networks were found to be as stable as the free enzyme at 65 °C for at least 4 hours. In the case of enzyme-BSA networks, primary dialysis of the free enzyme and BSA caused the removal of most of the impurities present in the commercial enzyme and crosslinking performed under vacuum at 65 °C, prevented the formation of a yellow colored by-product that changes the fabric color.

The performance of modified and unmodified enzymes was compared on textile materials such as viscose in hopes that the concept of using a loosely associated enzyme-polymer/enzyme-BSA networks might prove advantageous. In the case of modified Gempil 4L, the bursting strength (tensile fiber strength) had increased. Analysis of SEM pictures of untreated, G(-) treated and G(+) treated also supports the results of fabric strength analysis. Native enzyme caused the surface polishing of the viscose fibers. On the other hand crosslinked enzyme did not caused the same effect on the fibers. A plausible scenario to rationalize this finding rested on the premise that these loosely associated enzymes would remain restricted to the surface as opposed to the example of native enzymes, which could more easily permeate into the fiber matrix and promote mechanical degeneration. It follows to reason that the routine use of this

strategy may represent a feasible alternative to minimize the loss of mechanical strength in textiles.

6.1.3 Effects of Mechanical Agitation and Incubation Time

100 rpm of mechanical agitation and 1 or 2 hours of enzymatic incubation was found to be suitable for the process. Increasing the mechanical agitation would cause an increase in the enzyme activity and this would eventually lead to the the loss of fabric strength due to the aggressive action of the enzymes. Prolonged incubation times would also have a similar effect since the fabric would be more prone to attack by cellulases due to the synergism of the cellulase components that is, as one cellulase component attacks the cellulose, more reducing ends would be produced. This would eventually attract other cellulase components to the region and prolonged incubation would possibly result in the diffusion of the enzyme components into the fibers more and combined action of the cellulases inside the fiber would cause a decrease in the fiber and fabric strength.

6.1.4 Effects of Different Treatments

Effects of different pretreatments and surfactants on the properties of viscose fabric were also examined. 10 % ethylene glycol pretreatment of the fabric caused a slight increase in the fabric strength in case of both CLV-2h and G(-) but seem to had almost no effect on the pillings. Treatment with a cellulase, hemicellulase and xylanase mixture, Econase CE also did not cause loss of fabric strength even for 2 and 4 hours incubation. Lipase pretreatment and after treatments appears to have different effects in terms of fabric strength. Lipase after treatment had a more positive effect since the bursting strength values for the viscose fabric were better. TritonX-100, Tween 20 and Tween 80 did not cause any macroscopic changes on the fabric surface by visual inspection. This is consistent with the data in the literature concerning the effects of surfactants. According to Mazutani et al.(2001), Tween 20 shows its effects at least after 4 hours of incubation and for Tween 80 this time is at least 10 hours [19].

6.2 Combined Desizing and Enzymatic Bleaching with Native and Immobilized GOx

6.2.1 Bleaching Overview

Bleaching occurs as a result of the oxidation of the chromophores by the hydroxyl radicals formed by the dissociation of H_2O_2 . Metal ions and metal oxides are known to have a negative effect on the whiteness levels of fabrics. Decomposition of H_2O_2 was known to be accelerated due to the presence of them. Metal ions can come from the fabric itself or it can be present in the water used for bleaching. Commercial stabilizers are used for two purposes. One of them is to prevent decomposition of H_2O_2 by buffering the bleaching bath and the other is to chelate metal ions from the bleaching bath. Temperature and pH are known to increase the dissociation of peroxide. Decomposition of peroxide reduces the efficiency of bleaching. For this purpose two types of bleaching was used throughout the experiments.

The starting material is also important for peroxide generation. The amount of size on the cotton fabric determines the peroxide levels. The cotton fabric used in the experiments was the one with lowest size amount in the market. If the size amount increases then the amount of glucose increases and this would also increase the amount of peroxide that would be produced by GOx.

Bleaching in the presence of a peroxide activator (Prestogen SP) allowed the bleaching process to be performed at neutral pHs and bleaching in the presence of a peroxide stabilizer allowed the bleaching to be performed at alkaline pHs without decomposition of H_2O_2 . The combination of two processes created a closed loop system where one process bath was used for two processes. Moreover, the use of glucose produced from desizing to generate H_2O_2 is an environmentally friendly alternative to the use of harsh chemicals. Use of enzymes instead of harsh chemicals would increase the costs, but recycling of the enzymes is possible by immobilizing them on solid supports. Repeatable use of the enzymes would lower the costs.

6.2.2 Effects of Different Supports on Peroxide Production and Fabric Whiteness

Immobilization studies on Al_2O_3 was not continued due to the formation of red-colored enzyme- Al_2O_3 conjugates. These colored conjugates affected the whiteness of the cotton fabric. Color formation was due to the reaction of APTS silanized support with glutaraldehyde.

Different types of supports were used to increase the activity of GOx and search for an enzyme-support system which can tolerate recycling without considerable activity loss.

The maximum loading capacity of the silica support was found to be 30 mg GOx per g support (Sample F). After that value the immobilization yields were reduced to 70 and 80 %. The peroxide production by immobilized GOx on silica supports was the best with sample F. Nearly 55 % activity against glucose substrate was retained upon immobilization but the yield reduced to nearly 30 % against the desizing liquor. This may be due to the presence of impurities coming from the cotton. These impurities may cause the decomposition of the peroxide which would lower the peroxide concentrations. Multiple use of the enzyme was not successful in terms of peroxide production. Peroxide production was reduced nearly by a half and this also affected the fabric whiteness by 3 units in the second use. The reductions in the activity of the immobilized GOx could be because of the pore limitations and diffusional limitations of the silica beads. Since GOx is a relatively larger protein-150 kDa the steric effects would be more pronounced.

GOx immobilized on Sepharose-4B was more active than the ones immobilized on silica support. This was probably due to the structure of the support. Sepharose-4B is the brand name of separating agarose matrix. The beads probably have a more porous structure than silica that would allow the binding of the enzymes without steric hindrances and the diffusional limitations would be less effective due to larger pore sizes. One major drawback is that these commercially available activated supports are

very expensive. Although the activities against the desizing liquor were better for these beads the whiteness indexes were not better.

Upon all modifications CLEAs were the best in terms of activity. 82 % of the original enzyme activity was retained at 0.3 % glutaraldehyde concentration. One problem with these aggregates is that they stick to the fabric surface and they are hard to recycle without the use of dialysis membranes. The activity of the aggregates increased with the increase in the concentration of the GOx. Moreover, the activity of the aggregates decreased by increasing the glutaraldehyde concentration. As the glutaraldehyde concentration increases, more crosslinks are introduced into the aggregate system and the aggregate complex becomes tighter and due to steric hindrances activity losses are expected.

It was observed that during bleaching process in the presence of Prestogen SP, most of the peroxide (over 50 %) left in the bleaching bath. This means that most of the peroxide can not be activated effectively. Better whiteness levels could be reached if all this peroxide was activated. One hypothesis relates this with the production of gluconic acid during bleaching. This gluconic acid acts as a stabilizer for peroxide during bleaching. Up to certain levels gluconic acid is thought to be helpful in preventing the rapid decomposition of peroxide but at higher concentrations it may also prevent the fabric from using the peroxide present in the bleaching bath.

6.2.3 Effects of Temperature, pH, Aeration and Mechanical Agitation

6.2.3.1 Temperature and pH

Native GOx (1.5 mg) which is equivalent to the one in sample F was used to treat the fabric (3.5 g) at temperatures ranging from 25 °C to 55 °C and at pH 4 and pH 7. The analysis was performed at every 10°C to see the effect of temperature and pH on H₂O₂ production. The best H₂O₂ generation was achieved at 55 °C, pH 4 and pH 7 against the fabric. On the other hand best peroxide generation was at 35 °C at pH 7 against glucose substrate for sample F. The temperature optimum and pH optimum were

different against different substrates. Basically the substrates were the same but impurities present in the desizing liquor probably interferes with the enzymatic mechanism of peroxide generation.

6.2.3.2 Aeration and Mechanical Agitation

Aeration during the enzymatic process is important since GOx produces H_2O_2 from glucose in the presence of O_2 . Aeration not only introduces O_2 to the system but also increases the dissolved oxygen in the liquor with mechanical agitation. Aeration of the system increased the peroxide generation from 255 mg/ml to 663 mg/ml. Glass beads used during the enzymatic and bleaching processes provided the mechanical action. Use of these beads also increased the peroxide production from 633 mg/ml to 744 mg/ml but the whiteness index of the fabrics did not improved (for NGOx). Moreover, use of fabric bags for the recycling of the enzymes also reduced H_2O_2 produced due to the diffusional limitations with respect to the free IGOx.

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The presence of seed coat fragments on the surface of cotton was another factor that affects the bleachability of the cotton fabric. Interaction of peroxide with the seed coat fragments would consume some of the peroxide from the bleaching bath. Mechanical agitation appears to be important for the removal of these seed coat fragments. Application of cellulase and xylanase based formulations would also be helpful in the removal of these fragments. Preliminary studies with Econase CE indicated that application of these enzymes during desizing reduced the whiteness levels.

CHAPTER 7

CONCLUSIONS

7.1 Biopolishing of Viscose Knitted Fabrics with Native and Crosslinked Cellulases

Incubation time, incubation temperature, mechanical agitation and pH were found to be very important parameters affecting the fabric strengths. The short term incubation of the viscose knitted fabric with native and crosslinked enzymes in the presence of different surfactants did not improve the fabric surface properties. With the commercial enzymes available in the market, pilling values of the knitted fabrics were not improved much. Enlargement of the cellulases by crosslinking prevented the loss of fabric strength but it did not improve biopolishing. Pure cellulases would be tried for the biopolishing processes. Use of cellulase components with different ratios would also be helpful for the determination of the optimum formulations against viscose knitted fabrics.

7.2 Combined Desizing and Bleaching with Native and Immobilized GOx

Combined desizing and biobleaching of cotton fabrics with immobilized GOx produced whiteness levels that are not appropriate for white textiles but is sufficient for further dyeing processes. The whiteness and peroxide levels reached by the native enzyme were better than the immobilized enzyme for all support types. Best retained activities were attained with Crosslinked Enzyme Aggregates (CLEA). Aeration during H₂O₂ production and mechanical agitation provided with shakers at 250 rpm and glass beads were found to increase the peroxide production as expected. Alkali bleaching in the presence of a stabilizer was found to be more efficient than bleaching in the

presence of a peroxide activator. The temperature and pH optima of the immobilized and free enzyme were found to exhibit different characteristics against 50 mM glucose and against the glucose present in the desizing liquor. The activity of the enzyme was halved upto 5 cycles of recycling. Entrapment of the immobilized enzymes inside fabric bags and dialysis membranes were found to be useful for the recycling. Biopretreatment of cotton fabrics were also known to have an effect on the bleachability of the cotton. Different pretreatments with pectinases, lipases and proteinases would be tried on the cotton fabric for enhancing the whiteness levels. Studies on CLEAs and different acrylic resins would be performed to have a more active and stable enzyme.

APPENDICES

APPENDIX A

EQUIPMENTS

Equipment	Brand Name/Model, Company
Autoclave	Certoclav, Table Top Autoclave CV-EL-12L, AUSTRIA
	Hirayama, Hiclave HV-110, JAPAN
Balance	Sartorius, BP211D, GERMANY
	Sartorius, BP221S, GERMANY
	Sartorius, BP610, GERMANY
	Schimidzu, Libror EB-3200 HU, JAPAN
Burette	Borucam, TURKEY
Centrifuge	Eppendorf, 5415C, GERMANY
	Eppendorf, 5415D, GERMANY
	Eppendorf, 5415R, GERMANY
	Hitachi, Sorvall Discovery 100 SE, USA
	Hitachi, Sorvall RC5C Plus, USA
	Kendro Lab. Prod., Heraeus Multifuge 3L, GERMANY
Dialysis Membrane	Sigma, Cellusept
Distilled Water	Millipore, Elix-S, FRANCE
	Millipore, MilliQ Academic, FRANCE
Electrophoresis	Biorad Inc., USA
Eppendorf tubes(1.5-2ml)	Eppendorf
Falcon tubes(15-50ml)	TPP
Freezer	-70 °C, Kendro Lab. Prod., Heraeus Hfu486 Basic, GERMANY
	-20 °C, Bosch, TURKEY
Glasswares	Schott Duran, GERMANY

Hybridization Oven	Model 1012, Biolab, TURKEY
Ice Machine	Scotsman Inc., AF20, USA
Incubator	Memmert, Modell 300, GERMANY
	Memmert, Modell 600, GERMANY
Lyophilizer	
Magnetic Stirrer	ARE Heating Magnetic Stirrer, VELP Scientifica, ITALY
	Microstirrer, VELP Scientifica, ITALY
Micropipette	Eppendorf
Microscope	Olympos
Microtiter Plates (96-well)	TPP
Microtiterplate reader	Model 680, BioRad,
Microvave oven	Bosch, TURKEY
Multitube rotator	Labline
pH-meter	FisherBrand
Pipettman	Hirschman Laborgate,
Power Supply	Biorad, PowerPac 300, USA
	Wealtec, Elite 300, USA
Refrigerator (+4°C)	Bosch, TURKEY
SDS-PAGE Gel Casting Apparatus	Biorad
SEM	Gemini 35 VP, Carl Zeiss, GERMANY
Shaker	Forma Scientific, Orbital Shaker 4520, USA
	C25HC Incubator shaker New Brunswick Scientific, USA
	GFL, Shaker 3011, USA
	New Brunswick Sci., Innova™ 4330, USA
Spectrophotometers	Schimadzu, UV-1208, JAPAN
	Schimadzu, UV-3150, JAPAN
	BioRad
Speed Vacuum	Savant, Speed Vac® Plus Sc100A, USA
	Savant, Refrigerated Vapor Trap RVT 400, USA
Tips	TPP
Thermal Heater	Bioblock Scientific
Thermomixer	Eppendorf
Water bath	Huber, Polystat cc1, GERMANY

APPENDIX B

SDS-PAGE Protocol

1. Assemble the gel casting apparatus, making sure that the sandwich of glass plates and spacers will make a good seal.
2. Prepare the Separating Gel solution according to the acrylamide concentration needed and vortex.

12% Separating Gel

	amount	Final concentration
30 % acryl/ 0.8 % bisacryl	2 ml	12 %
ddH ₂ O	2.285 ml	45.7 %
3M Tris·Cl pH 8.9	625 µl	3.75 mM
10% SDS	50 µl	0.1%
10% APS	37.5 µl	0.075 %
TEMED	2.5 µl	0.0005 %
Total Volume	5 ml	

3. Load the apparatus with 4.5 mL of the Separating Gel solution.
4. Top with ~1 mL of Isoamyl alcohol to isolate the polymerization from oxygen.
5. After polymerization, pour off the Isoamyl alcohol, and rinse with distilled water.
6. Remove any water droplets from the inside of the casting apparatus with Whatman paper or a paper towel. Insert the comb for the stacking gel.
7. Prepare the Stacking Gel solution. Vortex.

Stacking Gel (5 % acrylamide)

	Amount	Final Concentration
30% acrylamide/ 0.8% bisacrylamide	425 µl	5 %
ddH ₂ O	1.92 ml	76.8 %
1M Tris·Cl pH 6.8	125 µl	50 mM
10% SDS	10 µl	0.1 %
10% APS	18.75 µl	0.075 %
TEMED	1.25 µl	0.05 %
Total Volume	2.5 ml	

12. Load the Stacking Gel solution, taking care not to introduce air bubbles around the comb (some bubbles can be removed by pipetting up and down).
13. Allow the Stacking Gel to polymerize completely (~45 minutes) before removing comb.
14. Prepare the samples:
 1. Dilute the protein sample 1:1 with 2x SDS Sample Buffer.
 2. Heat the samples and the molecular weight standards for 5 minutes at 100°C.
15. Remove the glass and gel sandwich from the casting apparatus.
16. Clip the sandwich to the electrophoresis apparatus. Carefully remove the comb from the gel and fill the top of the apparatus with 1x SDS Electrophoresis Buffer.
17. Using a 20-gauge needle, flush the wells with buffer.
18. Carefully load the samples into the bottom of the wells using a flat-tipped pipette tip.
19. Fill the bottom of the electrophoresis apparatus with 1x SDS Electrophoresis Buffer and connect the apparatus to the power supply.
20. Run the gel at 100 V until the dye enters the separating gel. Then increase the voltage to 150 V.
21. When the dye reaches the bottom of the separating gel, turn off the power supply,

and remove the gel sandwich.

22. Carefully open the sandwich by using one of the spacers to pry the plates apart.
23. Gently keep the stacking gel and place the separating gel in a small plastic container for staining.
24. Cover the gel with staining solution. Shake gently for ~ 30 minutes.
25. Pour off the staining solution and cover the gel with the wash solution. Destain for at least 2 hours. (It is usually necessary to change the wash solution at least once)
26. The gel can be stored in water or dried down between sheets of cellulose on a drying frame.

APPENDIX C

CNBr-Activated Sepharose 4B Product Information



3050 Spruce Street
Saint Louis, Missouri 63103 USA
Telephone 800-325-5832 • (314) 771-576
Fax (314) 286-7828
email: techserv@sial.com
sigma-aldrich.com

Product Information

CYANOGEN BROMIDE ACTIVATED MATRICES

Sigma Prod. Nos. C9210, C9142, C9267

Exact replacement for Product Code 16777

CAS NUMBER: N/A

Sigma offers three very similar cyanogen bromide-activated agarose matrices for use in making agarose beads for affinity chromatography. The general procedure used to couple ligands is the same for each, and is found on p. 3. General information and references are given on pages 1-2.

Product Number	C9210 ¹	C9142	C9267
Matrix Description	4% agarose cross linked	Sepharose ² 4% agarose	Sepharose ² 6% agarose macrobeads
Bead diameter	40-165 µm	40-165 µm	200-300 µm
Binding capacity per mL gel	≥ 35 mg BSA ³	~30-40 mg chymotrypsinogen	≥ 20 mg chymotrypsinogen ⁴
Appearance	white powder	white powder	white powder

STORAGE / STABILITY AS SUPPLIED:

These products are stable for at least eighteen months at 2-8°C if kept very dry; gradually over five years, about 50% loss in binding capacity can be expected.³ The activated resins are extremely moisture-sensitive.

ABOUT THE AGAROSE RESINS:

Processed agarose has a primary structure consisting of alternating residues of D-galactose and 3,6-anhydrogalactose. These sugars provide an uncharged hydrophilic matrix.

Cross-linked agarose is usually preferred over the non-cross-linked variety for most affinity applications that require harsh activation or usage conditions. Unfortunately, the added stability gained by cross-linking results in 30-50% loss of potential sites (consumed in the chemistry of cross-linking).⁷ The addition of cross-links to stabilize beaded agarose does not reduce porosity significantly. Larger beads allow higher flow rate; Sepharose 6 MB beads are significantly larger (200-300 µm diameter) than most beaded agaroses (40-165 µm).

CYANOGEN BROMIDE ACTIVATED MATRICES Sigma Prod. Nos. C9210, C9142, C9267

ABOUT THE ACTIVATION / COUPLING:

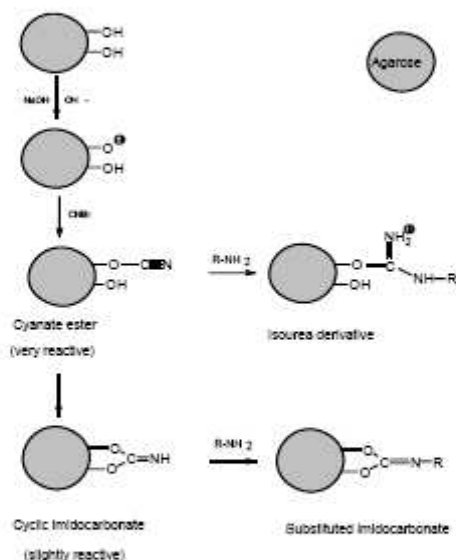
Cyanogen bromide in base reacts with -OH groups on agarose to form cyanate esters or imidocarbonates. These groups react readily with primary amines under very mild conditions; the net result is a covalent coupling of a ligand to the agarose matrix. The preferred resultant structure is an imidocarbonate, which has no net charge.

Why CNBr activation? The advantages:

- a) Many matrices contain -OH groups.
- b) The pH conditions needed for coupling are mild enough for many sensitive biomolecules.
- c) The procedure is relatively simple and reproducible.
- d) The coupling works for large and small ligands-although for very small ligands, a spacer may be used to reduce steric hindrance.
- e) the Sigma products are *already CNBr-activated*, so only need to be swollen, rinsed and added to coupling buffer.

The disadvantages:

- a) CNBr is highly toxic and sensitive to oxidation - most researchers prefer to use these *pre-activated* resins.
- b) The isourea bond formed between activated support and amine-ligand is somewhat unstable, so a small but constant leakage of coupled ligand may occur. Isourea derivatives may also act as weak anion exchangers, causing nonspecific binding, especially when small ligands are immobilized.⁷



REFERENCES:

1. C9210 replaces C1150; both produced by Sigma, but C9210 has higher binding capacity and better swelling stability.
2. Sepharose is a trademark of Pharmacia.
3. Sigma quality control or production department.
4. Supplier information.
5. *Affinity Chromatography - Principles and Methods* (Pharmacia Inc.), p. 15.
6. Hermanson, G.T., Mallia, A.K. and Smith, P.K., *Immobilized Affinity Ligand Techniques* (Academic Press, 1992), p. 53-58.
7. *Ibid.*, p. 6-9.

CYANOGEN BROMIDE ACTIVATED MATRICES
Sigma Prod. Nos. C9210, C9142, C9267

ADDITIONAL REFERENCES:

General information on procedure and usage: *Methods in Enzymology*, 34B, 77-102 (1974); 104, 3-11 (1984).

Preparation of DNA-agarose and use in enzyme purification: *Eur. J. Biochem.*, 54, 411 (1975).

Hermanson, G.T., Mallia, A.K. and Smith, P.K., *Immobilized Affinity Ligand Techniques* (Academic Press, 1992), p. 258.

Preparing antibody resins: *Affinity Chromatography: A Practical Approach*, eds. Dean, Johnson and Middle (Oxford Press, 1985), p. 31-34, 119. Hermanson, *ibid.*, p. 223.

GENERAL PREPARATION / USE:^{3,5,6}

Although numerous references can be found in the literature for use with specific proteins (antibodies, enzymes, etc.) or nucleic acids, this protocol is written for general purposes.

1. Dissolve protein to be coupled in 0.1 M NaHCO₃ buffer containing 0.5 M NaCl, pH 8.3-8.5 (about 5-10 mg protein per mL of gel). Note: Other buffers can be used, but avoid amine-containing buffers such as Trizma or other nucleophiles (buffers with amino groups) which will react with the binding sites.
2. Wash and swell cyanogen-bromide activated resin in cold 1 mM HCl for at least 30 minutes. A total of 200 ml per gram of dry gel is added in several aliquots. Remove the supernatant (contains lactose) by gentle suction in a Büchner or suction funnel between successive additions. Note: Lactose is necessary to stabilize the beads during freeze-drying, but it will interfere with binding if present during coupling. The use of HCl preserves the activity of the reactive groups which hydrolyze at high pH.
3. Wash the resin with distilled water, 5 - 10 column volumes, then wash the resin with the NaHCO₃/NaCl coupling buffer (5 ml per gram dry gel) and immediately transfer to a solution of the ligand in coupling buffer. Note: The reactive groups hydrolyze in basic solution!
4. Mix protein with gel for 2 hours at room temperature or overnight at 4°C. Use a paddle stirrer or end-over-end mixer, but not a magnetic stir bar (which may grind beads).
5. Wash away unreacted ligand using NaHCO₃/NaCl coupling buffer described above.
6. Block unreacted groups with either 1 M ethanolamine or 0.2 M glycine, pH 8.0 for 2 hours at room temperature or 16 hours at 4°C.
7. Wash extensively to remove the blocking solution, first with basic coupling buffer at pH ~ 8.5, then with acetate buffer (0.1 M, pH 4) containing NaCl (0.5 M).
8. Complete this wash cycle of high and low pH buffer solutions four or five times.
9. If the resin is to be used immediately, equilibrate it in buffer. If not, store the resin in 1.0 M NaCl at 2-8°C with a suitable bacteriostat.

03/03

Sigma warrants that its products conform to the information contained in this and other Sigma/Aldrich publications. Purchaser must determine the suitability of the product(s) for their particular use. Additional terms and conditions may apply. Please see reverse side of the invoice or packing slip.

APPENDIX D

a)

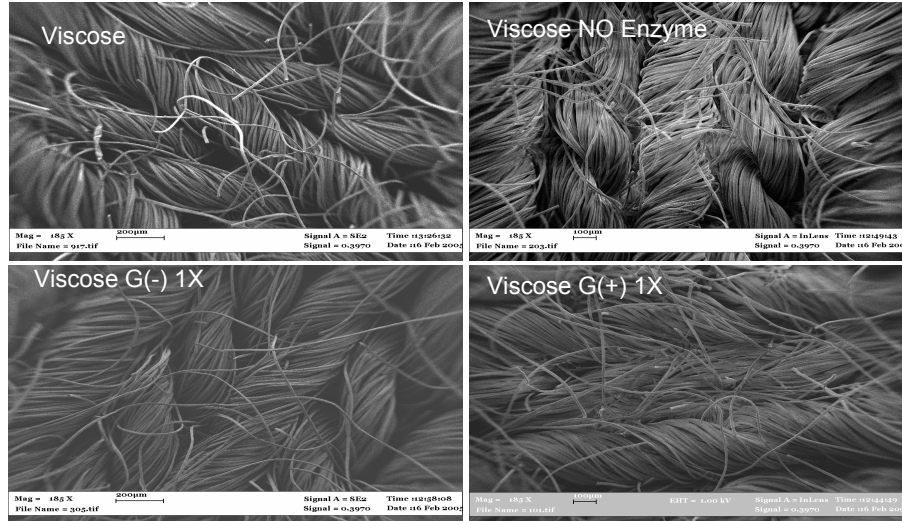
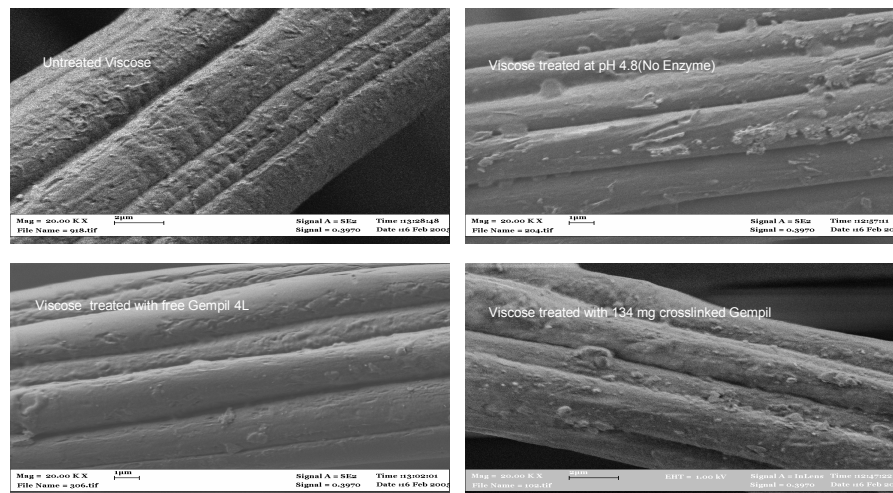


Figure 8.1: SEM pictures of untreated viscose fabric, viscose fabric incubated in 0.05M pH 4.8 NaOAc buffer, viscose fabric treated with native enzyme (G(-)) and crosslinked enzyme(G(+)) having the same protein concentrations. (Treatment conditions: 2 hours treatment at 400 rpm L.R.=1:15 at 55 °C) under a) 185X magnification b) 20000X magnification.

b)



a)

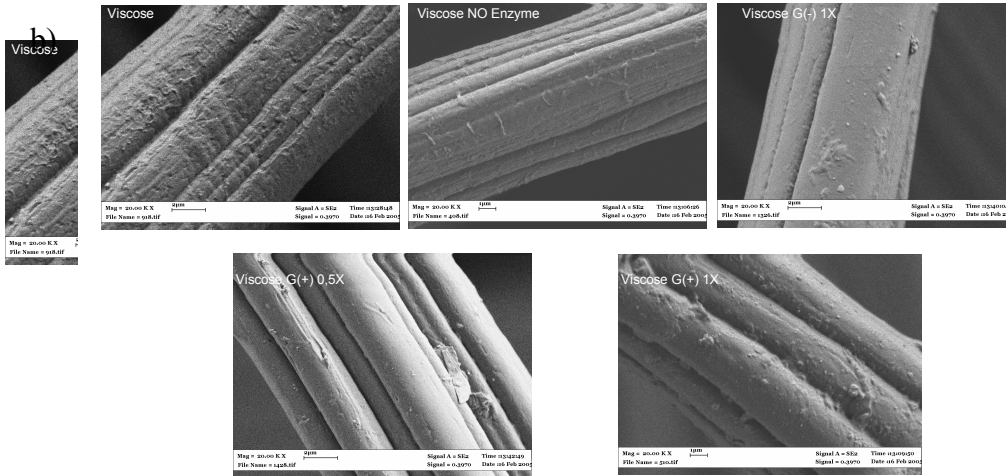
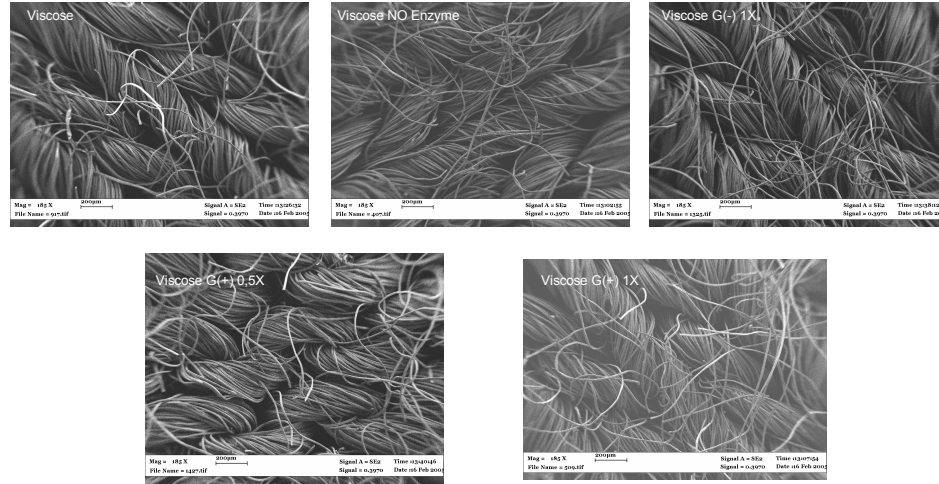


Figure 8.2: SEM pictures of untreated viscose fabric, viscose fabric incubated in 0.05M pH 4.8 NaOAc buffer, viscose fabric treated with native enzyme (G(-)) and crosslinked enzyme(G(+)) having the same protein concentrations G(-)1X and G(+)0.5X and having the same activities G(-)1X and G(+)1X (Treatment conditions:3 hours treatment at 400 rpm L.R.=1:15 at 55 °C) under a)185X magnification b) 20000X magnification.

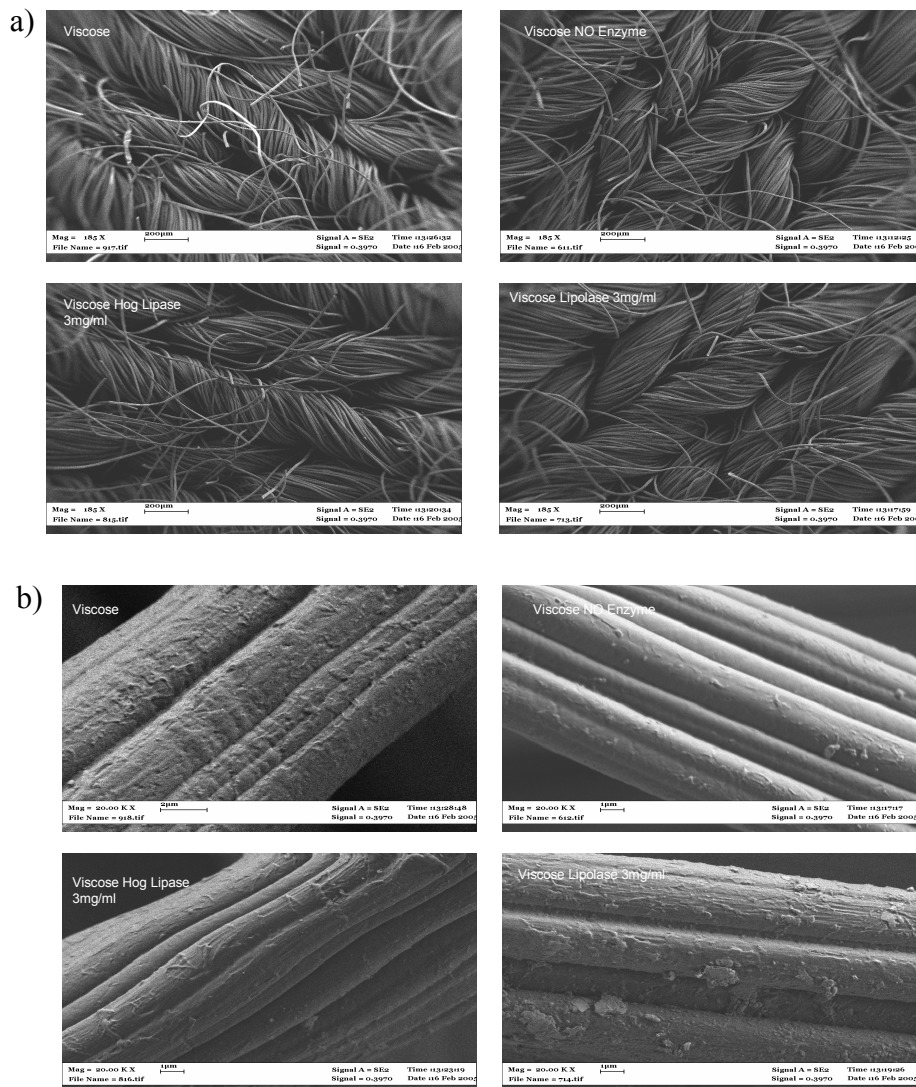


Figure 8.3: SEM pictures of untreated viscose fabric, viscose fabric incubated in 0.05M pH 4.8 NaOAc buffer, viscose fabric treated with Hog lipase (3 mg/ml) and Lipolase (3 mg/ml) (Treatment conditions: 2 hours treatment at 400 rpm L.R.=1:15 at 55 °C) under a) 185X magnification b) 20000X magnification.

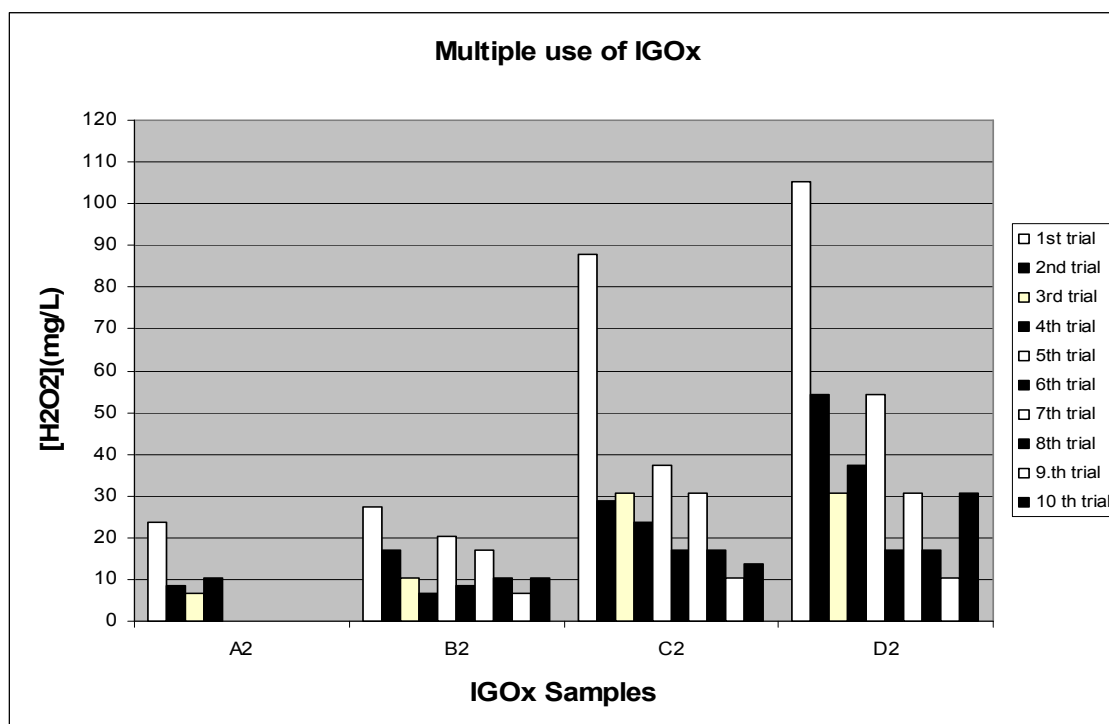


Figure 8.4: Multiple use of IGOx samples A, B, C and D upto 10 cycles and the resultant H_2O_2 production.

APPENDIX E

Amplex Red Assay Product Information



Product Information

Revised: 01-October-2004

Amplex[®] Red Glucose/Glucose Oxidase Assay Kit (A22189)

Quick Facts

Storage upon receipt:

- -20°C
- Desiccate
- Protect from light

Abs/Em of reaction product: 571/585 nm

Introduction

The Amplex[®] Red Glucose/Glucose Oxidase Assay Kit (A22189) provides a sensitive one-step method for detecting glucose or glucose oxidase. The Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) is a colorless, stable and extremely versatile peroxidase substrate. Because peroxidase- and glucose oxidase-mediated reactions can be coupled,^{1,2} it is possible to measure glucose oxidase activity or the release of glucose by any glucosidase enzyme — for instance, β -glucosidase and glucocerebrosidase — in either a continuous or discontinuous assay. This assay should also be very useful for quantitation of glucose levels in foods, fermentation media and bodily fluids.

In the assay, glucose oxidase reacts with D-glucose to form D-gluconolactone and H₂O₂. In the presence of horseradish peroxidase (HRP), the H₂O₂ then reacts with the Amplex Red reagent in a 1:1 stoichiometry to generate the red-fluorescent oxidation product, resorufin.³ Resorufin has absorption and fluorescence emission maxima of approximately 571 nm and 585 nm, respectively (Figure 1), and because the extinction coefficient is high (54,000 cm²M⁻¹), the assay can be performed either fluoro-

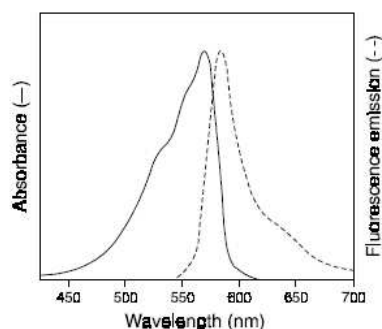


Figure 1. Normalized absorption and fluorescence emission spectra of resorufin, the product of the Amplex Red reaction.

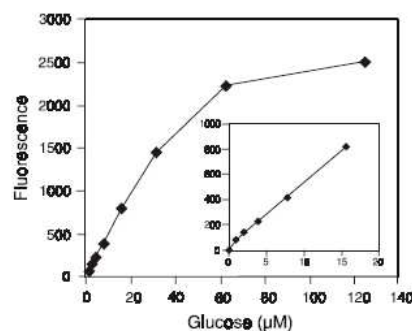


Figure 2. Detection of glucose using the Amplex Red Glucose/Glucose Oxidase Assay Kit (A-22189). Reactions containing 50 μ M Amplex Red reagent, 0.1 U/mL HRP, 1 U/mL glucose oxidase and the indicated amount of glucose in 50 mM sodium phosphate buffer, pH 7.4, were incubated for 30 minutes at room temperature. Fluorescence was then measured with a fluorescence microplate reader using excitation at 530 ± 12.5 nm and fluorescence detection at 590 ± 17.5 nm. Background fluorescence (201 arbitrary units), determined for a no-glucose control reaction, has been subtracted from each value. The inset shows the sensitivity and linearity of the assay at low levels of glucose.

metrically or spectrophotometrically. Furthermore, at these long wavelengths, there is little interference from autofluorescence found in most biological samples. With the Amplex Red Glucose/Glucose Oxidase Assay Kit, we have detected as little as 3 μ M D-glucose (Figure 2) and 0.05 mU/mL glucose oxidase (Figure 3).

Materials

Kit Contents

- **Amplex Red reagent** (MW = 257, Component A, blue cap), five vials, each containing 154 μ g of reagent
- **Dimethylsulfoxide (DMSO)**, anhydrous (Component B, green cap), 700 μ L
- **5X Reaction Buffer** (Component C, white cap), 28 mL of 0.25 M sodium phosphate, pH 7.4
- **Horseradish peroxidase** (Component D, yellow cap), 10 U, where 1 unit (U) is defined as the amount of enzyme that will form 1.0 mg purpurogallin from pyrogallol in 20 seconds at pH 6.0 at 20°C
- **Glucose oxidase** (Component E, orange cap), 100 U, where 1 unit (U) is defined as the amount that will oxidase 1.0 μ mole of β -D-glucose to D-gluconolactone and H₂O₂ per minute at pH 5.1 and 35°C
- **D-Glucose** (MW = 180, Component F, black cap), ~1 g

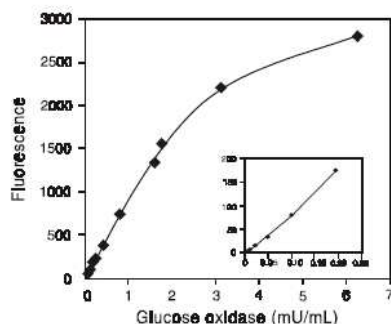


Figure 3. Detection of glucose oxidase using the Amplex Red Glucose/Glucose Oxidase Assay Kit (A-22189). Reactions containing 50 μ M Amplex Red reagent, 0.1 U/mL HRP, 50 mM glucose and the indicated amount of glucose oxidase in 50 mM sodium phosphate buffer, pH 7.4, were incubated for 30 minutes at room temperature. Fluorescence was measured with a fluorescence microplate reader using excitation at 530 ± 12.5 nm and fluorescence detection at 590 ± 17.5 nm. Background fluorescence (19 arbitrary units), determined for a no-glucose oxidase control reaction, has been subtracted from each value. The inset shows the assay's sensitivity at low levels of glucose oxidase.

- **Hydrogen peroxide (H_2O_2)** (Component G, red cap), 200 μ L of a stabilized ~3% solution; the actual concentration is indicated on the label

Each kit provides sufficient reagents for approximately 500 assays using either a fluorescence or absorbance microplate reader and reaction volumes of 100 μ L per assay.

Storage and Handling

Upon receipt, the kit should be stored frozen at -20°C , protected from light. Stored properly, the kit components should remain stable for at least six months. Allow reagents to warm to room temperature before opening vials. The Amplex Red reagent is somewhat air sensitive. Once a vial of Amplex Red reagent is opened, the reagent should be used promptly. PROTECT THE AMPLEX RED REAGENT FROM LIGHT.

Experimental Protocol

The following procedure is designed for use with a fluorescence or absorbance microplate reader. For use with a standard fluorometer, volumes must be increased accordingly.

Please note the following restrictions on the use of the Amplex Red reagent. The Amplex Red reagent is unstable in the presence of thiols such as dithiothreitol (DTT) and 2-mercaptoethanol. The final concentration of DTT or 2-mercaptoethanol in the reaction should be no higher than 10 μ M. The Amplex Red reagent is also unstable at high pH (>8.5). Furthermore, the absorption and fluorescence of the reaction product, resorufin, are pH-dependent. Below the pK_a (~ 6.0), the absorption maximum shifts to ~ 480 nm and the fluorescence quantum yield is markedly lower. For these reasons, the reactions should be performed at pH 7–8. The provided reaction buffer, pH 7.4, is recommended.

Stock Solution Preparation

1.1 Prepare a 10 mM stock solution of Amplex Red reagent. Allow one vial of Amplex Red reagent (Component A, blue cap) and DMSO (Component B, green cap) to warm to room temperature. Just prior to use, dissolve the contents of the vial of Amplex Red reagent in 60 μ L of DMSO. Each vial of Amplex Red reagent is sufficient for approximately 100 assays, with a final reaction volume of 100 μ L per assay.

1.2 Prepare 1X Reaction Buffer. Add 4 mL of 5X Reaction Buffer (Component C, white cap) to 16 mL of deionized water (dH_2O). This 20 mL volume of 1X Reaction Buffer is sufficient for approximately 100 assays of 100 μ L each with a 10 mL excess for making stock solutions.

1.3 Prepare a 10 U/mL stock solution of horseradish peroxidase (HRP). Dissolve the contents of the vial of HRP (Component D, yellow cap) in 1 mL of 1X Reaction Buffer. After the assay, any remaining unused solution should be divided into single-use aliquots and stored frozen at -20°C .

1.4 Prepare a 100 U/mL glucose oxidase stock solution. Dissolve the contents of the vial of glucose oxidase (Component E, orange cap) in 1.0 mL of 1X Reaction Buffer. This stock solution should be stored frozen at -20°C .

1.5 Prepare a 400 mM (72 mg/mL) glucose stock solution. Weigh out a portion of glucose (Component F, black cap), and dissolve it in the appropriate amount of 1X Reaction Buffer

1.6 Prepare a 20 mM H_2O_2 working solution. Dilute the ~3% H_2O_2 stock solution (Component G, red cap) into the appropriate volume of 1X Reaction Buffer. The actual concentration of H_2O_2 is indicated on the label. For instance, a 20 mM H_2O_2 working solution can be prepared from a 3.0% (0.88 M) H_2O_2 stock solution by diluting 22.7 μ L of 3.0% H_2O_2 into 977 μ L of 1X Reaction Buffer. Please note that although the ~3% H_2O_2 stock solution has been stabilized to slow degradation, the 20 mM H_2O_2 working solution prepared in this step will be less stable and should be used promptly.

Glucose Assay

The following protocol describes the assay of glucose in a total volume of 100 μ L per microplate well. The volumes recommended here are sufficient for ~100 assays. The kit provides sufficient material for ~500 assays.

2.1 Prepare a glucose standard curve. Dilute the appropriate amount of the 400 mM glucose stock solution (prepared in step 1.5) into 1X Reaction Buffer to produce glucose concentrations of 0 to 200 μ M, each in a volume of 50 μ L. Be sure to include a no-glucose control. Final glucose concentrations will be twofold lower (e.g., 0 to 100 μ M).

2.2 If no standard curve is to be used, prepare positive and negative controls. For a glucose-positive control, dilute the 400 mM glucose stock solution (prepared in step 1.5) to 200 μ M in 1X Reaction Buffer. For an H_2O_2 -positive control, dilute the 20 mM H_2O_2 working solution (prepared in step 1.6) to 10 μ M

in 1X Reaction Buffer. For a negative control, use 1X Reaction Buffer without H_2O_2 .

2.3 Dilute the glucose-containing samples in 1X Reaction Buffer. A volume of 50 μL will be used for each reaction. A variable dilution will be required depending on the total glucose present in the sample. In the first trial the samples should be serially diluted to determine the optimal amount of sample for the assay. Note that extremely high levels of glucose (e.g., 500 μM , final concentration) can produce lower fluorescence than moderately high levels (e.g., 100 μM), because excess H_2O_2 resulting from the reaction of glucose with glucose oxidase can oxidize the reaction product, resorufin, to nonfluorescent resazurin.

2.4 Load the samples. Pipet 50 μL of the standard curve samples, controls and experimental samples into individual wells of a microplate.

2.5 Prepare a working solution of 100 μM Amplex Red reagent, 0.2 U/mL HRP and 2 U/mL glucose oxidase. Mix the following:

- 50 μL of 10 mM Amplex Red reagent stock solution (prepared in step 1.1)
- 100 μL of 10 U/mL HRP stock solution (prepared in step 1.3)
- 100 μL of 100 U/mL glucose oxidase stock solution (prepared in step 1.4)
- 4.75 mL of 1X Reaction Buffer

This 5 mL volume is sufficient for ~100 assays. Note that the final concentration of each component will be twofold lower in the final reaction volume.

2.6 Begin the reactions. Add 50 μL of the Amplex Red reagent/HRP/glucose oxidase working solution to each microplate well containing the standards, controls and samples.

2.7 Incubate the reactions. Incubate at room temperature for 30 minutes, protected from light. Because the assay is continuous (not terminated), fluorescence or absorbance may be measured at multiple time points to follow the kinetics of the reactions.

2.8 Measure the fluorescence or absorbance. Use a microplate reader equipped for excitation in the range of 530–560 nm and fluorescence emission detection at ~590 nm, or for absorbance at ~560 nm (see Figure 1).

2.9 Correct for background fluorescence or absorbance. For each point, subtract the value derived from the no-glucose control.

Glucose Oxidase Assay

The following protocol describes the assay of glucose oxidase in a total volume of 100 μL per microplate well. The volumes here are sufficient for ~100 assays. The kit provides sufficient material for ~500 assays.

3.1 Prepare a glucose oxidase standard curve. Dilute the appropriate amount of 100 U/mL glucose oxidase stock solution (prepared in step 1.4) into 1X Reaction Buffer (prepared in step 1.2) to produce glucose oxidase concentrations of approximately

0 to 10 mU/mL glucose oxidase, each in a volume of 50 μL . Be sure to include a no-glucose oxidase control. Please note that final glucose oxidase concentrations will be twofold lower (e.g., 0 to 5 mU/mL).

3.2 If no standard curve is to be used, prepare positive and negative controls. For a glucose oxidase-positive control, dilute the 100 U/mL glucose oxidase stock solution (prepared in step 1.4) to 10 mU/mL in 1X Reaction Buffer. For an H_2O_2 positive control, dilute the 20 mM H_2O_2 working solution to 10 μM in 1X Reaction Buffer. For a negative control, use 1X Reaction Buffer without H_2O_2 .

3.3 Dilute the glucose oxidase-containing samples in 1X Reaction Buffer. A volume of 50 μL will be used for each reaction. A variable dilution will be required depending on the total glucose oxidase present in the sample. In the first trial, the samples should be serially diluted to determine the optimal amount of sample for the assay. Note that extremely high levels of glucose oxidase (e.g., 50 mU/mL, final concentration) can produce lower fluorescence than moderately high levels (e.g., 10 mU/mL), because excess H_2O_2 resulting from the reaction of glucose with glucose oxidase can oxidize the reaction product, resorufin, to nonfluorescent resazurin.

3.4 Load the samples. Pipet 50 μL of the standard curve samples, controls and experimental samples into individual wells of a microplate.

3.5 Prepare a working solution of 100 μM Amplex Red reagent, 0.2 U/mL HRP and 100 mM glucose. Mix the following:

- 50 μL of 10 mM Amplex Red reagent stock solution (prepared in step 1.1)
- 100 μL of 10 U/mL HRP stock solution (prepared in step 1.3)
- 1.25 mL of 400 mM glucose stock solution (prepared in step 1.5)
- 3.60 mL of 1X Reaction Buffer

This 5 mL volume is sufficient for ~100 assays. Note that the final concentration of each component will be twofold lower in the final reaction volume.

3.6 Begin the reactions. Add 50 μL of the Amplex Red reagent/HRP/glucose working solution to each microplate well containing the standards, controls and samples.

3.7 Incubate the reactions. Incubate at room temperature for 30 minutes, protected from light. Because the assay is continuous (not terminated), fluorescence or absorbance may be measured at multiple time points to follow the kinetics of the reactions.

3.8 Measure the fluorescence or absorbance. Use a microplate reader equipped for excitation in the range of 530–560 nm and fluorescence emission detection at ~590 nm (see Figure 1), or for absorbance at ~560 nm.

3.9 Correct for background fluorescence or absorbance. For each point, subtract the value derived from the no-glucose oxidase control.

References

1. Proc SPIE-Intl Soc Opt Eng 3606, 119 (1999); 2. Anal Biochem 253, 162-168 (1997); 3. J Immunol Methods 202, 133 (1997).

Product List

Current prices may be obtained from our Web site or from our Customer Service Department.

Cat #	Product Name	Unit Size
A22189	Amplex® Red Glucose/Glucose Oxidase Assay Kit "500 assays"	1 kit
A12222	Amplex® Red reagent (10-acetyl-3,7-dihydroxyphenoxazine)	5 mg
A22177	Amplex® Red reagent "packaged for high-throughput screening"	10 x 10 mg
A36006	Amplex® UltraRed reagent	5 x 1 mg
R363	resorufin, sodium salt "reference standard"	100 mg

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REFERENCES

1. Aleshin A.E., Feng P., Honzatko R.B., Reilly P.J., *Crystal Structure and Evolution of a Prokaryotic Glucoamylase*, Journal of Molecular Biology (2003) 327, 61–73.
2. Azevedo, H., Bishop, D., Cavaco-Paulo, A., *Effects of agitation level on the adsorption, desorption, and activities on cotton fabrics of full length and core domains of EGV (Humicola insolens) and CenA (Cellulomonas fimi)*. Enzyme and Microbial Technology 27 (2000), 325-329.
3. Bhat, M.K., Bhat S., *Cellulose degrading enzymes and their potential industrial applications*. Biotechnology Advances 15 (1997), 583-620.
4. Bhat, M.K., Bhat, S., *Cellulases and related enzymes in biotechnology*. Biotechnology Advances 18 (2000), 355-383.
5. Bisswanger, H., Practical Enzymology, (2004) Wiley-VCH Verlag GmbH & Co. KgaA, Weinheim.
6. Bower, B., Clarkson, K., Collier, K., Kellis, J., Kelly, M. and Larenas, E. 1998. *High molecular weight Trichoderma cellulase*. WO 98/15619, PCT/US97/18402.
7. Bradford, M.M., *A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of dye-protein binding*. Anal. Biochem 72 (1976), 248-254.
8. Bright H. J., Appleby, M., J. Biol. Chem. (1969) 244, 3625-3634.
9. Buschle-Diller, G., Yang, X.D., Yamamoto, R., *Enzymatic Bleaching of Cotton Fabric with Glucose Oxidase*, Textile Research Journal 71(2) (2001), 388-394.
10. Castanon, M., Wilke, C.R., *Effects of the Surfactant Tween 80 on Enzymatic Hydrolysis of Newspaper*. Biotechnology and Bioengineering 23 (1981), 1365-1372.

11. Cho, Y.K., Bailey, J.E., *Immobilization of enzymes on activated carbon: Properties of immobilized glucoamylase, glucose oxidase, and gluconolactone*. Biotechnol. Bioeng. 20 (1978) 1651-1665.
12. Ciechańska, D., Struszczyk, H., Miettinen-Oinonen, A., Strobin, G., *Enzymatic Treatment of Viscose Fibres Based Woven Fabric*. FIBRES & TEXTILES in Eastern Europe 6 (2002), 60-63.
13. Colom, X., Carrillo, F., *Crystallinity changes in lyocell and viscose-type fibres by caustic treatment*. European Polymer Journal 38 (2002), 2225-2230.
14. Colowick, S.P., Kaplan, N.O., ed Mosbach, K., *Immobilized Enzymes, Methods in Enzymology Volume 44*, 1976. Academic Press, Inc. (London) Ltd.
15. Divne, C., Stahlberg, J., Reinikainen, T., Ruohonen, L., Pettersson, G., Knowles, J.K., Teeri, T.T., Jones, T.A, *The three-dimensional crystal structure of the catalytic core of cellobiohydrolase I from Trichoderma reesei*. Science 265 (1994), 524-528.
16. Edt. Bickerstaff, G., *Immobilization of Enzymes and Cells, Methods in Biotechnology*, 1997 Humana Press Inc. Totowa, New Jersey.
17. Edt. Brown, R.M., *Cellulose and Other Natural Polymer Systems: Biogenesis, Structure and Degradation*, Plenum Press New York and London.
18. Feitkenhauer, H., Fischer, D., Fah, D., *Microbial desizing using starch as model compound: enzyme properties and desizing efficiency*. Biotechnol. Prog. 19(3) (2003), 874-879.
19. Gibson Q. H., Swoboda B. E. P., Massey V., *Kinetics and Mechanism of Action of Glucose Oxidase*. J. Biol. Chem. 239 (1964), 3927-3934.
20. Gruno, M., Valjamae, P., Pettersson, G., Johansson, G., *Inhibition of the Trichoderma reesei Cellulases by Cellobiose Is Strongly Dependent on the Nature of the Substrate*. Biotechnology and Bioengineering, 86(5) (2004), 504-511.
21. Gupta R., Gigras P., Mohapatra H., Goswami V.K., Chauhan B., *Microbial α -amylases: a biotechnological perspective*, Process Biochemistry 38 (2003) 1599-1616.
22. Halliwell, G. and Vincent, R., *The action of cellulose and its derivatives of a purified 1,4- β -glucanase from Trichoderma koningii*. Biochem. J. 199 (1981), 409-417.

23. Hecht H.J., Kalisz H.M., Hendle J., Schmid R. D., Schomburg D. , *Crystal Structure of Glucose Oxidase from Aspergillus niger Refined at 2.3 Å Resolution* J. Mol. Biol. (1993) 229, 153-172.
24. Heikinheimo, L., Buchert, J. Synergistic effects of *Trichoderma reesei* cellulases on the properties of knitted cotton fabric. *Textile Research Journal* 71, 672-677 (2001).
25. Heinkimo L., *Trichoderma reesei* cellulases in processing of cotton. VTT Publications 483, Espoo 2002. 77 p. +app. 37 p.
26. <http://pd-beta.rcsb.org/pdb/search.do?newSearch=yes&inputQuickSearch=1CF3&IMAGE.x=0&IMAGE.y=0&IMAGE=Search>, Release Date: 26 March 1999.
27. <http://www.degussa.co.nz/downloads/Application%20-%20Textile.pdf>, © 2000 Degussa Peroxide Ltd.
28. <http://www.engr.utk.edu/mse/pages/Textiles/Cotton%20fibers.htm>. Hegde, R.R., Dahiya, A., Kamath, M.G. (Gao, X., Jangala, P.K.) *Cotton Fibers* (updated) 2004.
29. <http://www.netorder.jp/maker/rakuto/textile/index.html>
30. <http://www.rcsb.org/pdb/cgi/explore.cgi?pid=245351121927768&pdbId=1AMY>, Release Date: 13 May 1995.
31. <http://www.rcsb.org/pdb/cgi/explore.cgi?pid=260501121928178&pdbId=1AC0>, Release Date: 07 July 1997.
32. <http://www.starch.dk/isi/starch/starch.htm>, Copyright © 1999 International Starch Institute, Science Park Aarhus, Denmark.
33. <http://www.food.reading.ac.uk/online/fs560/topic2/t2a/t2a.htm>. Last updated 17th December 1996 by Dr. R A Rastall.
34. J.D. McCarter, S.G. Withers, *Mechanisms of enzymatic glycoside hydrolysis*, *Curr. Opin. Struct. Biol.* 4 (1994) 885-892.
35. Kaar, W.E., Holtzaple, M.T., *Benefits from Tween During Enzymic Hydrolysis of Corn Stover*. *Biotechnology and Bioengineering* 59(4) (1998), 419-427.
36. Kleywegt, G.J. *The Crystal Structure of the Catalytic Core Domain of Endoglucanase I from Trichoderma reesei at 3.6Å Resolution, and a*

- Comparison with Related Enzymes*. Journal of Molecular Biology 272 (1997), 383-397.
37. Kumar, A., Yoon, M., Purtell, C., *Optimizing the Use of Cellulase Enzymes in Finishing Cellulosic Fabrics*. Textile Chemist and Colorist 29 (1997), 37-42.
 38. Lamed, R., Bayer, E.A., *The cellulosome of Clostridium thermocellum*. Adv. Appl. Microbiol. 33 (1988), 1-46.
 39. Lange, N.K., Liu J., Husain, P., Condon, B., *Cotton BioPreparation – a new Enzymatic Concept*. 2001-23105-01 Dec 2001 © Novozymes A/S. http://www.novozymes.com/library/Downloads/Products_and_solutions/2001-23105.pdf
 40. Lenting, H.B.M., Warmoeskerken, M.M.C.G., *Guidelines to come to minimized tensile strength loss upon cellulase application*. Journal of Biotechnology 89 (2001), 227-232.
 41. Liming, X., Xueliang, S., *High-yield cellulase production by Trichoderma reesei ZU-02 on corn cob residue*. Bioresource Technology 91 (2004), 259-262.
 42. Lopez-Serrano, P., Cao, L., van Rantwijk, R., Sheldon, R.A., *Cross-linked enzyme aggregates with enhanced activity: application to lipases*. Biotechnology Letters 24: 1379–1383, 2002.
 43. Medve, J., Karlsson, J., Lee, D., Tjerneld, F., *Hydrolysis of Microcrystalline Cellulose by Cellobiohydrolase I and Endoglucanase II from Trichoderma reesei: Adsorption, Sugar Production Pattern, and Synergism of the Enzymes*. Biotechnology and Bioengineering 59 (1998), 621-634.
 44. Miettinen-Oinonen, A., Paloheimo, M., Lantto, R., Suominen, P., *Enhanced production of cellobiohydrolases in Trichoderma reesei and evaluation of the new preparations in biofinishing of cotton*. Journal of Biotechnology 116 (2005), 305-317.
 45. Miller, G. L., *Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar*. Anal. Chem. 31(3) (1959), 426-428.
 46. Mizutani, C., Sethumadhavan, K., Howley, P., Bertoniere, N., *Effect of a Nonionic Surfactant on Trichoderma cellulase treatments of regenerated cellulose and cotton yarns*. Cellulose 9 (2002), 83-39.
 47. Nemeth, A. *Increasing the thermal stability of cellulase C using rules learned from thermophilic proteins: a pilot study*. Biophysical Chemistry 132 (2002), 229-241.

48. Ng, T.B., *Peptides and proteins from fungi*. *Peptides* 25, 1055-1073.
49. Novozymes. Enzymes at work. .
http://www.novozymes.com/library/Downloads/Publications/Enzymes_2004.pdf
50. Ooshima, H., Sakata, M., Harano, Y., *Enhancement of Enzymatic Hydrolysis of Cellulose by surfactant*. *Biotechnology and Bioengineering* 28 (1986), 1727-1734.
51. Reinikainen, T., Teleman, O., Teeri, T.T., *Effects of pH and High Ionic Strength on the Adsorption and Activity of Native and Mutated Cellobiohydrolase I from Trichoderma reesei*. *Proteins: structure, Function and Genetics* 22 (1995), 392-403.
52. Robert X., Haser R., Gottschalk T.E., Ratajczak F., Driguez H., Svensson B., Aghajari N., *The Structure of Barley α -Amylase Isozyme 1 Reveals a Novel Role of Domain C in Substrate Recognition and Binding: A Pair of Sugar Tongs*, *Structure* 11(2003) 973–984.
53. Sandgren, M.C., *Structural and biochemical studies of GH family 12 cellulases: improved thermal stability, and ligand complexes*. *Progress in Biophysics and Molecular Biology*, article in press (2005).
54. Sauer J., Sigurskjold B.W., Christensen U., Frandsen T.P., Mirgorodskaya E., Harrison M., Roepstorj P., Svensson B., *Review, Glucoamylase: structure/function relationships, and protein engineering*, *Biochimica et Biophysica Acta* 1543 (2000) 275-293.
55. Sheldon, R. A., Lopez-Serrano, R., Cao, L., van Rantwijk, F., *Cross-linked enzyme aggregates with enhanced activity: application to lipases*. *Biotechnology Letters* 24 (2002), 1379-1383.
56. Shin, Y., Son, K., Yoo, D.I. *Structural Changes in Tencel by Enzymatic Hydrolysis*. *Journal of Applied Polymer Science* 76(2000), 1644-1651.
57. Silva R.N., Asquieri E.R., Fernandes K.F., *Immobilization of Aspergillus niger glucoamylase onto a polyaniline polymer*, *Process Biochemistry* (2004) (article in press).
58. Tzanov, T., Costa, S.A., Guebitz, G.M., Cavaco-Paulo, A., *Hydrogen peroxide generation with immobilized glucose oxidase for textile bleaching*. *Journal of Biotechnology* 93 (2002), 87–94.

59. Videbaek, T. and Andersen, L. D. 1993. A Process for defuzzing and depilling cellulosic fabrics. PCT int. Appl. WO 9320278.
60. Vinzant T. B., Adney, W.S., Decker, S. R., Baker, J. O., Kinter, M. T., Sherman, N. E., Fox, J.W., Himmel, M.E., *Fingerprinting Trichoderma reesei Hydrolases in a Commercial Cellulase Preparation*. Applied Biochemistry and Biotechnology 91–93 (2001).
61. Weibel, M. K., Bright, H. J., J. Biol. Chem. (1971) 246, 2734-2744.
62. Witt S., Wohlfahrt G., Schomburg D., Hecht H., Kalisz H.M., *Conserved arginine-516 of Penicillium amagasakiense glucose oxidase is essential for the efficient binding of b-D-glucose*, Biochem. J. (2000) 347, 553-559.
63. www.CAZY.org
64. Zhang, Y.H., Lynd, L.R., *Toward an Aggregated Understanding of Enzymatic Hydrolysis of Cellulose: Noncomplexed Cellulase Systems, Review*. Biotechnology and Bioengineering 88(7) (2004), 797-824.